10-16-08 fw DAC

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE :	U.S. Patent No. 6,835,809
ISSUED:	December 28, 2004
TO:	CHUAN-FA LIU, *et al.*
FOR:	THROMBOPOIETIC COMPOUNDS
FROM:	SERIAL NO. 09/422,838
FILED:	October 22, 1999

I hereby certify that this correspondence is being deposited with the United States Postal Service as Express Mail, Airbill No. EL995022051US in an envelope addressed to: Mail Stop Patent Extension Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450

Dated: October 15, 2008

Signature: Juan Quintero

Mail Stop Patent Extension
Commissioner for Patents
P.O. Box 1450
Arlington, VA 22313-1450

REQUEST FOR EXTENSION OF PATENT TERM UNDER 35 U.S.C. §156

Dear Sirs,

Transmitted herewith under 37 C.F.R. 1.730(c) are the application papers of Amgen Inc., dated October 15, 2008, for extension of U.S. Patent No. 6,835,809 under 35 U.S.C. §156, based on the regulatory review period for Nplate™ (romiplostim), together with two duplicate copies as required under 37 C.F.R. §1.740(b) and two additional duplicate copies of the application pursuant to M.P.E.P. §2753, for a total of four copies and one original.

U.S. Patent No. 6,835,809, granted to Chaun-Fa Liu, *et al.* on December 28, 2004, was assigned to Kirin-Amgen by virtue of assignments recorded in the United States Patent and Trademark Office on October 22, 1999, at Reel 010336, Frame 0930; and on June 28, 2007, at Reel 19494, Frame 0101. Amgen Inc., effective July 1, 2005, is the licensee of Kirin-Amgen with respect to U.S. Patent No. 6,835,809.

As set forth under 37 C.F.R. §1.20(j), please charge the sum of $1,120.00 to Deposit Account No. 13-2855 for the filing of this application for extension of patent

10/16/2008 HLE333 00000029 132855 6835809
01 FC:1457 1120.00 DA

term. Also, please charge any underpayment, or any additional fee that may be required, or credit any overpayment, to Deposit Account No. 13-2855. Two copies of this paper are enclosed.

Respectfully submitted,
for Amgen Inc.

Date: October 15, 2008

Joseph A Williams, Jr. (Reg. No. 38,659)
Attorney for Licensee
Marshall Gerstein & Borun LLP
233 South Wacker Drive
6300 Sears Tower
Chicago, IL 60606-6357

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE :	U.S. Patent No. 6,835,809
ISSUED:	December 28, 2004
TO:	CHUAN-FA LIU, *et al.*
FOR:	THROMBOPOIETIC COMPOUNDS
FROM:	SERIAL NO. 09/422,838
FILED:	October 22, 1999

I hereby certify that this correspondence is being deposited with the United States Postal Service as Express Mail, Airbill No. EL995022051US in an envelope addressed to: Mail Stop Patent Extension Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450

Dated: October 15, 2008

Signature: Juan Quintero

Mail Stop Patent Extension
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

REQUEST FOR EXTENSION OF THE TERM OF UNITED STATES PATENT NO. 6,835,809 UNDER 35 U.S.C. §156 FOR NPLATE™ (ROMIPLOSTIM)

Dear Sirs,

Amgen Inc., a corporation organized and existing under the laws of the State of Delaware, and having a place of business at One Amgen Center Drive, Thousand Oaks, California, 91320-1799, United States of America, represents that it is the licensee of Kirin-Amgen (effective July 1, 2005).of Letters Patent of the United States No. 6,835,809 granted to Chuan-Fa Liu, *et al.* on October 22, 1999, for Thrombopoietic Compounds. Kirin-Amgen, by virtue of assignments recorded in the United States Patent and Trademark Office (hereinafter referred to as "the Patent Office") on October 22, 1999, at Reel 010336, Frame 0930; and on June 28, 2007, at Reel 119494, Frame 0101, is assignee of U.S. Patent No. 6,835,809.

Pursuant to the provisions of 37 C.F.R. §1.730, Applicant hereby applies for an extension of the term of Patent No. 6,835,809 under 35 U.S.C. §156 of 818 days, based on the materials set forth herein and in the accompanying papers.

In the materials which follow herein, numbered paragraphs (1) through (15) correspond to paragraphs (1) through (15) of 37 C.F.R. §1.740(a).

(1) The approved product is Nplate™ (romiplostim). Nplate™ is a novel engineered therapeutic fusion protein with attributes of both peptides and antibodies, but is distinct from each. Nplate™ works similarly to thrombopoietin ("TPO"), a natural protein in the body. Nplate™ activates intracellular transcriptional pathways leading to increased platelet production via the TPO receptor (also known as "cMpl"). The peptibody molecule contains two identical single-chain subunits, each consisting of human immunoglobulin IgG1 Fc domain, covalently linked at the C-terminus to a peptide containing two thrombopoietin receptor-binding domains. Romiplostim has no amino acid sequence homology to endogenous TPO. Romiplostim is produced by recombinant DNA technology in *Escherichia coli (E coli)*.

Molecular Weight:

Nplate™ is a homodimer of a 269 amino acid polypeptide. The molecular weight of the dimer is 60.1 kilodaltons.

Structural Formula:

The amino acid sequence is from the amino terminus to the carboxy terminus in Nplate™. The CAS registry number for romiplostim is 267639-76-9.

```
MDKTHTCPPC PAPELLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSHE 50
DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVVSVLTVL HQDWLNGKEY 100
KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSRDELT KNQVSLTCLV 150
KGFYPSDIAV EWESNGQPEN NYKTTPPVLD SDGSFFLYSK LTVDKSRWQQ 200
GNVFSCSVMH EALHNHYTQK SLSLSPGKGG GGGIEGPTLR QWLAARAGGG 250
GGGGGIEGPT LRQWLAARA                                  269
```

We note that the term "product," for purposes of patent term extension for a drug product, is defined as "the active ingredient of . . . a new drug, antibiotic drug, or human biological product . . . including any salt or ester of the active ingredient, as a single entity or in combination with another active ingredient." 35 U.S.C. §156(f)(2).

(2) Nplate™ was subject to regulatory review under Section 351 of the Public Health Service Act, 42 U.S.C. §262.

(3) Nplate™ received permission for commercial marketing or use under section Section 351 of the Public Health Service Act, 42 U.S.C. §262, on August 22, 2008.

(4) The active ingredient in Nplate™ is romiplostim, an engineered therapeutic fusion protein produced by recombinant DNA technology in *Escherichia coli* (*E coli*). That active ingredient has not been previously approved for commercial marketing or use under the Federal Food, Drug and Cosmetic Act, the Public Health Service Act or the Virus-Serum-Toxin Act.

(5) This application is being submitted within the sixty day period permitted for its submission pursuant to 37 C.F.R. §1.720(f). The last day on which this application could be submitted is October 21, 2008.

(6) The patent for which an extension is being sought is identified as follows:

Inventors: Chuan-Fa Liu, Ulrich Feige, and Janet C. Cheetham
Patent No.: 6,835,809
For: Thrombopoietic Compounds
Issued: December 28, 2004
Expires: October 22, 2019

(7) A copy of U.S. Patent No. 6,835,809, the patent for which an extension is being sought, is attached hereto as EXHIBIT A.

(8) One maintenance fee payment for U.S. Patent No. 6,835,809 was made to keep the patent in force beyond four years from its issue date (a copy of the receipt of such payment is included herewith as EXHIBIT B). Pursuant to 37 C.F.R. §1.740(a)(8), Applicants submit a copy of any Certificate of Correction for U.S. Patent No. 6,835,809 (EXHIBIT C).

(9) Patent No. 6,835,809 claims the approved product. Claims 1 and 3 read on the approved product.

Nplate™ is a compound that binds to the Mpl receptor and has the general formula:

F_c-L-TMP_1-$(L_1)_n$-TMP_2,

wherein Fc is an immunoglobulin constant region, L and L_1 are linkers, and TMP_1 and TMP_2 are peptides

Claim 1 of Patent No. 6,835,809 reads on NplateTM™ because:

Nplate™ is a compound that binds to the Mpl receptor and comprises the general structure TMP_1-$(L_1)_n$-TMP_2 wherein TMP_1 and TMP_2 are each peptides having the same amino acid sequence IEGPTLRQWLAARA, designated SEQ ID NO:1, separated by a linker.

(10) The relevant dates and information pursuant to 35 U.S.C. §156(g) in order to enable the Secretary of Health and Human Services to determine the applicable regulatory review period are as follows:

- An exemption under subsection (i) of Section 505 of the Federal Food, Drug and Cosmetic Act became effective for Nplate™ on 23 April 2002, following receipt and review by the Food and Drug Administration ("FDA") of an Investigational New Drug ("IND") Application, identified by FDA as BB-IND 10205, on April 19, 2002 (copies of FDA letters acknowledging receipt and effective date of the IND are included herewith as EXHIBITS D and E, respectively).
- A Biologics License Application ("BLA") under Section 351 of the Public Health Service Act for Nplate™ was submitted on 23 Oct 2007. The BLA was assigned the number 125268/0.
- BLA No. 125268/0 was approved on 22 Aug 2008(copies of FDA letter approving BLA No. 125268/0 and the FDA-approved product package insert are included herewith as EXHIBITS F and G, respectively).

(11) A brief description of the significant activities undertaken by the marketing applicant during the applicable regulatory review period with respect to the approved product and the significant dates applicable to such activities follows:

The IND for this drug was submitted on 20 Dec 2001. It was evaluated by FDA and a letter was issued by FDA dated 19 Apr 2002 granting the permission to proceed with clinical study contingent upon a specific protocol amendment. Subsequently, the protocol amendment was submitted and the IND became effective as of 23 Apr 2002. Amgen conducted a series of clinical investigations of this drug. On 23 Nov 2004, FDA granted fast track designation to this product. The BLA was submitted 23 Oct 2007 and approved on 22 Aug 2008.

The following chart identifies significant events and communications of substance with the FDA concerning this product:

Date	Regulatory Event
20 Dec 2001	Submission of IND (BB-IND 10205)
16 Jan 2002	FDA Letter Acknowledging Receipt of IND and Assignment of No. 10205
21 Feb 2002	FDA Letter Placing IND on Clinical Hold for Insufficient Information to Evaluate Clinical Risk
25 Feb 2002	FDA Fax Placing IND on Clinical Hold for Insufficient Information to Evaluate Clinical Risk
20 Mar 2002	Submission of Additional Information to IND (Response to Clinical Hold)
19 Apr 2002	FDA Letter Acknowledging Review of IND, Removing Clinical Hold, and Granting Approval Contingent Upon Submission of Protocol Amendment
23 Apr 2002	Submission of Protocol # 20000137, Amendment 1
7 May 2002	Letter from FDA Advising about Clinical Trials Database Requirements
9 Dec 2002	Submission of Request for Orphan Drug Status
27 Mar 2003	Letter from FDA Qualifying Nplate™ for Orphan Drug Status
1 Apr 2003	Response to FDA CMC Questions (original IND)

18 Dec 2003	End of Phase I (Clarification of CMC questions) Meeting with FDA
28 Sept 2004	Submission of Request for Fast Track Designation
23 Nov 2004	Letter from FDA granting Fast Track Status
23 Nov 2004	End of Phase II Meeting with FDA
18 Mar 2005	Submission to Request Special Protocol Assessment (Protocol # 20030105 and # 20030212)
2 May 2005	Letter from FDA, Results of Special Protocol Assessment (Protocol # 20030105 and # 20030212)
21 June 2005	Type B (CMC) End of Phase II Meeting with FDA
13 June 2006	Type C Meeting with FDA
9 Jan 2007	Type A Meeting with FDA
22 May 2007	Clinical Pre-BLA Meeting with FDA
24 May 2007	CMC Pre-BLA Meeting with FDA
23 Oct 2007	Submission of BLA 125268/0
23 Oct 2007	Submission of Request for FDA Priority Review Status
10 Dec 2007	Letter from FDA Granting Priority Review Status
12 Mar 2008	ODAC Advisory Board Meeting
27 Mar 2008	Submission of Risk Management Plan (RMP)
3 Apr 2008	Letter from FDA Extending Amgen's PDUFA Date to 23 July 2008
18 Apr 2008	Submission of US PI and Draft PMC/PMR
12 Aug 2008	Submission of Final Risk Evaluation Mitigation Strategy (REMS)
22 Aug 2008	Amgen receives FDA Approval Letter

(12) In the opinion of the Applicant, U.S. Patent No. 6,835,809 is eligible for an extension under 35 U.S.C. §156. The length of extension claimed is 818 days.

The length of extension of term of Patent No. 6,835,809 of 818 days claimed by the Applicant was determined according to the provisions of 37 C.F.R. §1.775 as follows:

- According to 37 C.F.R. §1.775(b), the length of extension is equal to the regulatory review period for the approved product, reduced as appropriate pursuant to paragraphs (d)(1) through (d)(6) of 37 C.F.R. §1.775.
- According to 37 C.F.R. §1.775(c), the regulatory review period is the sum of: (A) the number of days in the period beginning on the date the exemption under subsection 505(i) of the Federal Food, Drug and Cosmetic Act became effective for the approved product and ending on the date the BLA was initially submitted under Section 351 of the Public Health Service Act; and (B) the number of days in the period beginning on the date the BLA was initially submitted under Section 351 of the Public Health Service Act and ending on the date the BLA was approved. The exemption under subsection 505(i) of the Federal Food, Drug and Cosmetic Act became effective on April 23, 2002; and the BLA was submitted on October 23, 2007, and approved on August 22, 2008. Hence the regulatory review period under 37 C.F.R. §1.775(c) is the sum of the period from April 23, 2002 to October 23, 2007 and from October 23, 2007 to August 22, 2008. This is the sum of 2313 days.
- According to 37 C.F.R. §1.775(d)(1)(i), the number of days in the regulatory review period which were on and before the date on which the patent issued must be subtracted. Patent No. 6,835,809 issued on December 28, 2004. Subtraction of the period on or before December 28, 2004 leaves a reduced regulatory review period from December 29, 2004 to October 23, 2007 and from October 23, 2007 to August 22, 2008. This is the sum of 1028 days and 304 days, which is 1332 days.
- 37 C.F.R. §1.775(d)(1)(ii) does not apply.
- According to 37 C.F.R. §1.775(d)(1)(iii), the regulatory review period must then be reduced by one-half of the days remaining in the period defined in 37 C.F.R. §1.775(c)(1). This is one-half of 1028 days, which, disregarding half days, is 514 days. After subtraction, this now leaves a reduced regulatory review period of 514 days plus 304 days, which is 818 days.
- According to 37 C.F.R. §1.775(d)(2), the reduced regulatory review period of 818 days must be added to the expiration date of Patent No. 6,835,809, i.e., October 22, 2019. This gives a date of January 17, 2022.
- According to 37 C.F.R. §1.775(d)(3), 14 years must be added to the date of approval of the approved product. This gives a date of August 22, 2022.
- According to 37 C.F.R. §1.775(d)(4), the earlier of these dates must be selected. The earlier date of these dates is January 17, 2022.
- The provisions of 37 C.F.R. §1.775(d)(5) apply to this application because Patent No. 6,835,809 issued after September 24, 1984. Pursuant to 37 C.F.R. §1.775(d)(5)(i), five years are added to the expiration date of Patent No. 6,835,809 (October 22 2019) giving a date of October 22, 2024. According to 37 C.F.R. §1.775(d)(5)(ii), the dates obtained pursuant to 37 C.F.R.

§1.775(d)(5)(i) and 37 C.F.R. §1.775(d)(4) are compared and the earlier date is selected. The date calculated according to 37 C.F.R. §1.775(d)(4) above is January 17, 2022. Therefore, the earlier of these dates is January 17, 2022. Applicant is entitled to an extension of term of Patent No. 6,835,809 until January 17, 2022, i.e., an extension of 818 days from the original expiration date of October 22, 2019.

- The provisions of 37 C.F.R. §1.775(d)(6) do not apply because Patent No. 6,835,809 issued on December 28, 2004, after September 24, 1984.

(13) Applicant acknowledges a duty to disclose to the Director of United States Patent and Trademark Office and the Secretary of Health and Human Services any information which is material to the determination of entitlement to the extension which is being sought to the term of Patent No. 6,835,809.

(14) The prescribed fee under 37 C.F.R. §1.20(j) for receiving and acting on this application for patent term extension is to be charged to Deposit Account No. 13-2855

(15) Please direct all inquiries and correspondence relating to this application for patent term extension as follows:

Joseph A. Williams, Jr.
Marshall, Gerstein & Borun LLP
233 South Wacker Drive
6300 Sears Tower
Chicago, Illinois 60606-6357
(312) 474-6300 telephone
(312) 474-0448 facsimile

Pursuant to 37 C.F.R. §1.740(b), two duplicate copies of these application papers are enclosed herewith. Pursuant to M.P.E.P. §2753, an additional two copies of the application are also enclosed herewith. Accordingly, a total of one original application for patent term extension of Patent No. 6,835,809 and four copies of the application are submitted herewith.

Applicant respectfully requests prompt and favorable action on the merits of this application for extension of the term of Letters Patent No. 6,835,809 of 818 days, based on the regulatory review period for Nplate™ (romiplostim).

Respectfully submitted,
for Amgen Inc.

Date: October 15, 2008

Joseph A. Williams, Jr. (Reg. No. 38,659)
Attorney for Licensee
Marshall Gerstein & Borun LLP
233 South Wacker Drive
6300 Sears Tower
Chicago, IL 60606-6357

EXHIBIT A

U.S. Patent No. 6,835,809

US006835809B1

(12) **United States Patent**
Liu et al.

(10) **Patent No.: US 6,835,809 B1**
(45) **Date of Patent: Dec. 28, 2004**

(54) **THROMBOPOIETIC COMPOUNDS**

(75) Inventors: **Chuan-Fa Liu**, Longmont, CO (US); **Ulrich Feige**, Newbury Park, CA (US); **Janet C. Cheetham**, Montecito, CA (US)

(73) Assignee: **Amgen Inc.**, Thousand Oaks, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/422,838**

(22) Filed: **Oct. 22, 1999**

Related U.S. Application Data

(60) Provisional application No. 60/105,348, filed on Oct. 23, 1998.

(51) **Int. Cl.**[7] **A61K 38/16**; C07K 7/00; C07K 14/00

(52) **U.S. Cl.** **530/324**; 530/324; 530/326; 530/327; 514/12; 514/13; 514/14

(58) **Field of Search** 530/300, 402, 530/328; 424/178.1; 514/44, 15

(56) **References Cited**

U.S. PATENT DOCUMENTS

3,691,016	A		9/1972	Patel
3,941,763	A		3/1976	Sarantakis
3,969,287	A		7/1976	Jaworek et al.
4,195,128	A		3/1980	Hildebrand et al.
4,229,537	A		10/1980	Hodgins et al.
4,247,642	A		1/1981	Hirohara et al.
4,289,872	A		9/1981	Denkewalter et al.
4,330,440	A		5/1982	Ayers et al.
4,925,673	A		5/1990	Steiner et al.
5,013,556	A		5/1991	Woodle et al.
5,229,490	A		7/1993	Tam
5,284,656	A		2/1994	Platz et al.
5,428,130	A		6/1995	Capon et al.
5,480,981	A		1/1996	Goodwin et al.
5,739,277	A		4/1998	Presta et al.
5,792,451	A		8/1998	Sarubbi et al.
5,869,451	A	*	2/1999	Dower et al. 514/13
5,932,546	A		8/1999	Barrett et al.
6,251,864	B1	*	6/2001	Dower et al. 514/13

FOREIGN PATENT DOCUMENTS

WO	WO 93/21259	10/1993
WO	WO 95/18858	7/1995
WO	WO 95/21919	8/1995
WO	WO 95/21920	8/1995
WO	WO 95/26746	10/1995
WO	WO 96/32478	10/1996
WO	WO 96/40189	12/1996
WO	WO 96/40750	12/1996
WO	WO 97/34631	9/1997
WO	WO 98/25965	6/1998

OTHER PUBLICATIONS

Abuchowski and Davis, Soluble Polymer–Enzyme Adducts, Enzymes as Drugs, Hocenberg and Roberts, eds., Wiley–Interscience, New York, NY, (1981), pp 367–383.

Adjei et al., Pharmaceutical Research 7:565–569 (1990).
Adjei et al., International Journal of Pharmaceutics 61:135–144 (1990).
Alexander et al., Blood 87:2162–2170 (1996).
Bartley et al., Cell 77:1117–1124 (1994).
Basser et al., Lancet 348:1279–81 (1996).
Braquet et al., Journal of Cardiovascular Pharmacology 13 (suppl.5): s. 143–146 (1989).
Capon, et al., Nature 337:525–531 (1989).
Chang et al., Journal of Biological Chemistry 270:511–517 (1995).
Choi et al., Blood 85:402–413 (1995).
Clackson, T. et al., Science 267:383–396 (1995).
Creighton, T.E., Proteins: Structure and Molecule Properties, W.H. Freeman & Co., San Francisco, pp. 70–86 (1983).
Cwirla, S.E. et al., Science 276:1696–1699 (1997).
Davis et al., Biochem. Intl. 10:395–404 (1985).
Debili, N. et al., Blood 85:391–401 (1995).
Debs et al., The Journal of Immunology 140:3482–3488 (1988).
de Sauvage et al., Nature 369:533–538 (1994).
Devlin, J.J. et al., Science 249:404 (1990).
Duncan, Nature, 332:563 (1988).
Ellison, J.W. et al., Nucleic Acids Res. 10:4071–4079 (1982).
Erickson et al., The Proteins, 3rd ed., vol. 2, pp. 257–527 (1976).
Finn et al., The Proteins, 3rd ed., vol. 2, pp. 105–253 (1976).
Fisher, C. et al., N. Engl. J. Med., 334:1697–1702 (1996).
Gurney et al., Blood 85:981–988 (1995).
Harvill et al., Immunotechnology, 1:95–105 (1995).
Hokom, M.M. et al., Blood 86:4486–4492 (1995).
Hubbard et al., Annals of Internal Medicine 111:206–212 (1989).
Jefferis et al., Immunology Letters, vol. 44, pp. 111–117 (1995).
Jefferis et al., Molecular Immunology, vol. 27, pp. 1237–1240 (1990).
Johnson et al., Biochemistry, vol. 37, pp. 3699–3710 (1998).
Kaushansky et al., Nature 369:568–571 (1994).
Kato et al., Journal of biochemistry 118:229–236 (1995).
Kay et al., Reviews —Research Focus, vol. 3, No. 8, pp. 370–378 (1998).
Kuter et al., Proc. Natl. Acad. Sci. USA 91:11104–11108 (1994).
Livnah, O. et al., Science 273:464–471 (1996).
Lok, S. et al., Nature 369:565–8 (1994).

(List continued on next page.)

Primary Examiner—Michael Borin
(74) *Attorney, Agent, or Firm*—Marshall, Gerstein & Borun LLP

(57) **ABSTRACT**

The invention relates to the field of compounds, especially peptides or polypeptides, that have thrombopoietic activity. The peptides and polypeptides of the invention may be used to increase platelets or platelet precursors (e.g., megakaryocytes) in a mammal.

3 Claims, 7 Drawing Sheets

OTHER PUBLICATIONS

Marshall, K., Modern Pharmaceutics, Edited by G. S. Banker and C. T. Rhodes Chapter 10, 1979.
Merrifield, in Chem. Polypeptides, pp. 335–361 (Katsoyannis and Panayotis eds. 1973).
Merrifield, J. Am. Chem. Soc. 85:2149 (1963).
Methia et al., Blood 82:1395–1401 (1993).
Newmark, et al., J. Appl. Biochem. 4:185–189 (1982).
Palacios, R. et al., Cell 41:727 (1985).
Rasko et al., Stem Cells 15:33–42 (1997).
Ravin et al., Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, PA 18042) pp. 1435–1712.
Sarmay, Molecular Immunology, vol. 29, No. 5, 633–639 (1992).
Scott, J.K. et al., Science 249:386 (1990).
Sheridan et al., Platelets, vol. 8, pp. 319–332 (1997).
Smith et al., J. Clin. Invest. 84:1145–1146 (1989).
Stewart and Young, Solid Phase Peptide Synthesis (1969)—Table of Contents only.
Syed et al., Nature, 395:5111 (1998).
Ulich et al., Blood 86:971–976 (1995).
Van Zee, K. et al., The Journal of Immunology, 156:2221–2230 (1996).
Vigon, I. et al., Proc. Natl. Acad. Sci. USA 89:5640–5644 (1992).
Wells, J. A. et al., Ann. Rev. Biochem. 65:609–634 (1996).
Wrighton, N.C., et al., Science 273:458–469 (1996).
Wrighton, N.C., et al., Nature Biotechnology 15:1261–1265 (1997).
Zeigler et al., Blood 84:4045–4052 (1994).
Zheng, X. et al., The Journal of Immunology, 154:5590–5600 (1995).

* cited by examiner

```
       ATGGACAAAACTCACACATGTCCACCTTGTCCAGCTCCGGAACTCCTGGGGGGACCGTCA
     1 ---------+---------+---------+---------+---------+---------+ 60
       TACCTGTTTTGAGTGTGTACAGGTGGAACAGGTCGAGGCCTTGAGGACCCCCCTGGCAGT

a        M  D  K  T  H  T  C  P  P  C  P  A  P  E  L  L  G  G  P  S  -

       GTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTC
    61 ---------+---------+---------+---------+---------+---------+ 120
       CAGAAGGAGAAGGGGGGTTTTGGGTTCCTGTGGGAGTACTAGAGGGCCTGGGGACTCCAG

a        V  F  L  F  P  P  K  P  K  D  T  L  M  I  S  R  T  P  E  V  -

       ACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTG
   121 ---------+---------+---------+---------+---------+---------+ 180
       TGTACGCACCACCACCTGCACTCGGTGCTTCTGGGACTCCAGTTCAAGTTGACCATGCAC

a        T  C  V  V  V  D  V  S  H  E  D  P  E  V  K  F  N  W  Y  V  -

       GACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACG
   181 ---------+---------+---------+---------+---------+---------+ 240
       CTGCCGCACCTCCACGTATTACGGTTCTGTTTCGGCGCCCTCCTCGTCATGTTGTCGTGC

a        D  G  V  E  V  H  N  A  K  T  K  P  R  E  E  Q  Y  N  S  T  -

       TACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTAC
   241 ---------+---------+---------+---------+---------+---------+ 300
       ATGGCACACCAGTCGCAGGAGTGGCAGGACGTGGTCCTGACCGACTTACCGTTCCTCATG

a        Y  R  V  V  S  V  L  T  V  L  H  Q  D  W  L  N  G  K  E  Y  -

       AAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCC
   301 ---------+---------+---------+---------+---------+---------+ 360
       TTCACGTTCCAGAGGTTGTTTCGGGAGGGTCGGGGGTAGCTCTTTTGGTAGAGGTTTCGG

a        K  C  K  V  S  N  K  A  L  P  A  P  I  E  K  T  I  S  K  A  -

       AAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACC
   361 ---------+---------+---------+---------+---------+---------+ 420
       TTTCCCGTCGGGGCTCTTGGTGTCCACATGTGGGACGGGGGTAGGGCCCTACTCGACTGG

a        K  G  Q  P  R  E  P  Q  V  Y  T  L  P  P  S  R  D  E  L  T  -

       AAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTG
   421 ---------+---------+---------+---------+---------+---------+ 480
       TTCTTGGTCCAGTCGGACTGGACGGACCAGTTTCCGAAGATAGGGTCGCTGTAGCGGCAC

a        K  N  Q  V  S  L  T  C  L  V  K  G  F  Y  P  S  D  I  A  V  -

       GAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGAC
   481 ---------+---------+---------+---------+---------+---------+ 540
       CTCACCCTCTCGTTACCCGTCGGCCTCTTGTTGATGTTCTGGTGCGGAGGGCACGACCTG
```

FIG. 1A

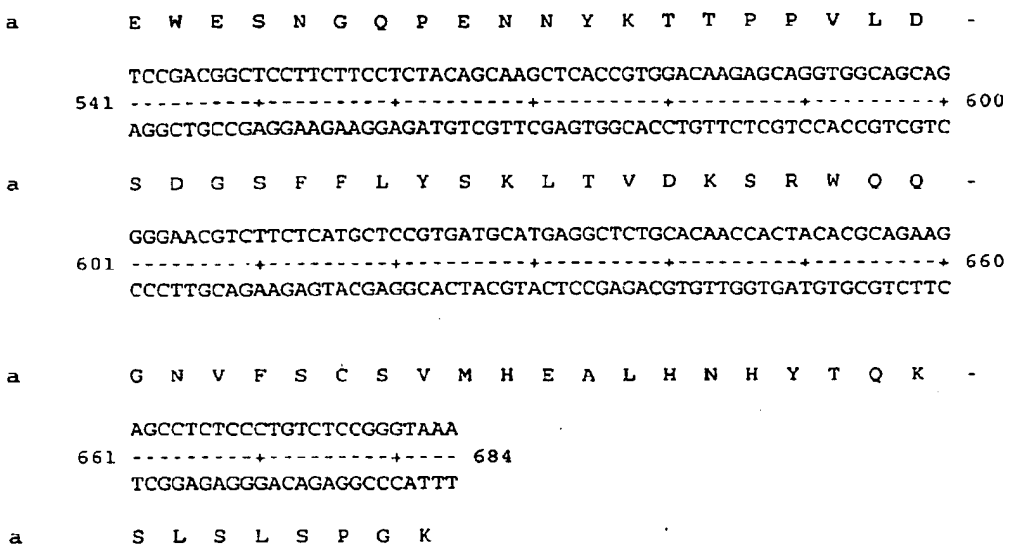

a E W E S N G Q P E N N Y K T T P P V L D -
TCCGACGGCTCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAG
541 ---------+---------+---------+---------+---------+---------+ 600
AGGCTGCCGAGGAAGAAGGAGATGTCGTTCGAGTGGCACCTGTTCTCGTCCACCGTCGTC
a S D G S F F L Y S K L T V D K S R W Q Q -
GGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAG
601 ---------+---------+---------+---------+---------+---------+ 660
CCCTTGCAGAAGAGTACGAGGCACTACGTACTCCGAGACGTGTTGGTGATGTGCGTCTTC
a G N V F S C S V M H E A L H N H Y T Q K -
AGCCTCTCCCTGTCTCCGGGTAAA
661 ---------+---------+---- 684
TCGGAGAGGGACAGAGGCCCATTT
a S L S L S P G K

FIG. 1B

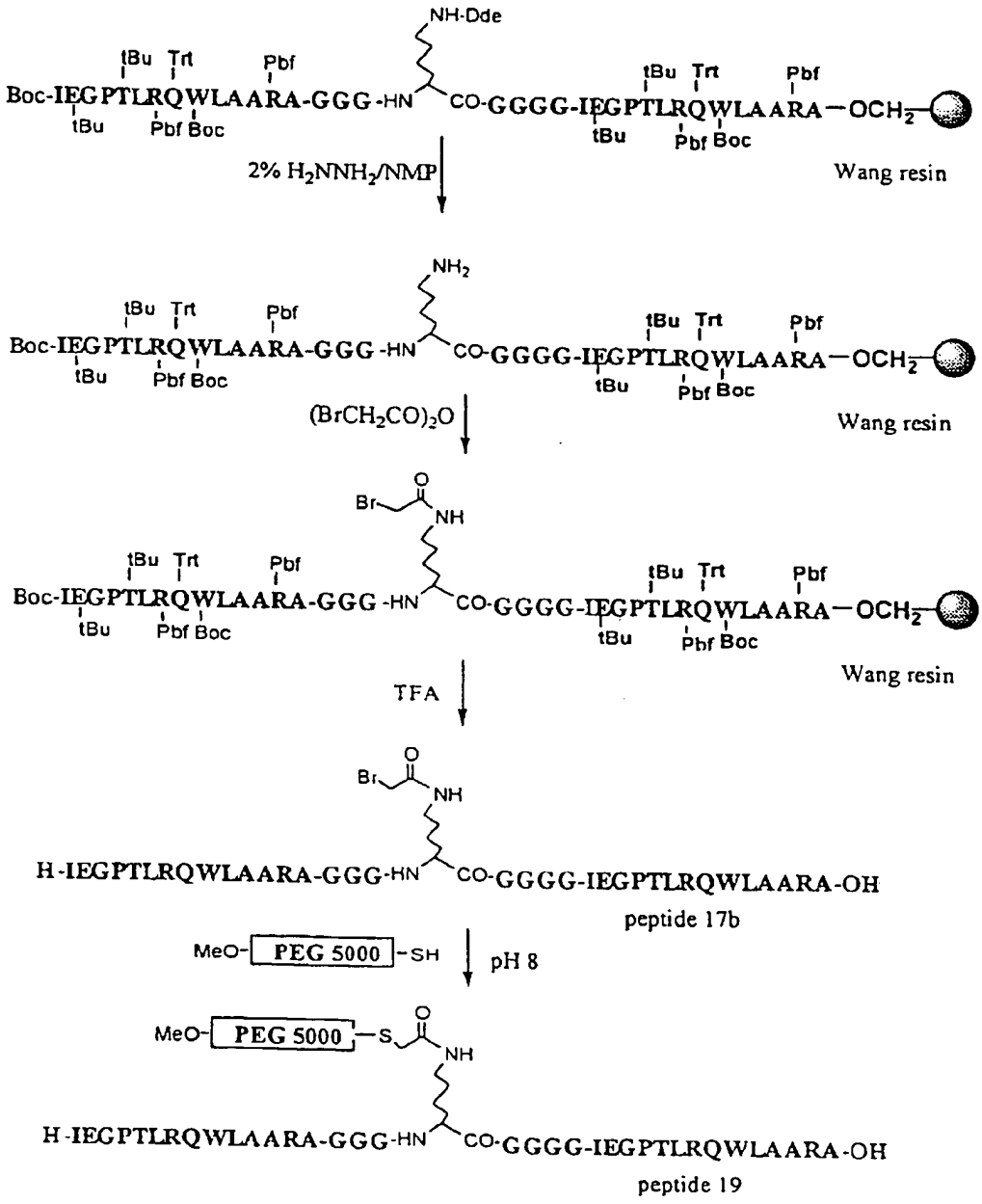

NH-Dde
tBu Trt Pbf
Boc-IEGPTLRQWLAARA-GGG-HN
CO-GGGG-IEGPTLRQWLAARA—OCH2
tBu Pbf Boc
2% H2NNH2/NMP
Wang resin
NH2
(BrCH2CO)2O
Br
NH
TFA
H-IEGPTLRQWLAARA-GGG-HN
CO-GGGG-IEGPTLRQWLAARA-OH
peptide 17b
MeO-PEG 5000-SH
pH 8
MeO-PEG 5000-S
peptide 19

FIG. 2

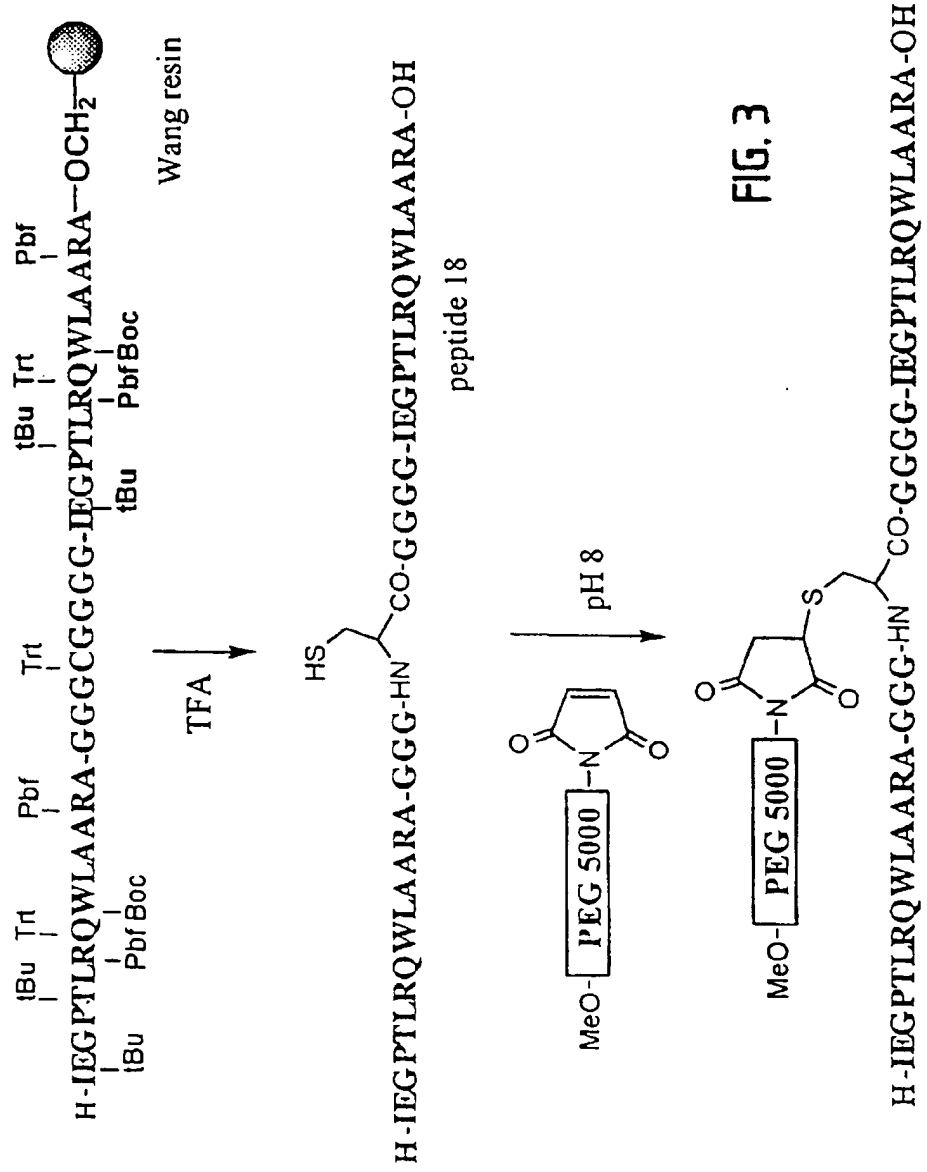

H-IEGPTLRQWLAARA-GGGCGGGG-IEGPTLRQWLAARA—OCH2
tBu Trt Pbf Trt tBu Trt Pbf
tBu Pbf Boc tBu PbfBoc
Wang resin
TFA
H-IEGPTLRQWLAARA-GGG-HN CO-GGGG-IEGPTLRQWLAARA-OH
HS
peptide 18
pH 8
MeO- PEG 5000 -N
MeO- PEG 5000 -N
S
H-IEGPTLRQWLAARA-GGG-HN CO-GGGG-IEGPTLRQWLAARA-OH
peptide 20

FIG. 3

FIG. 4

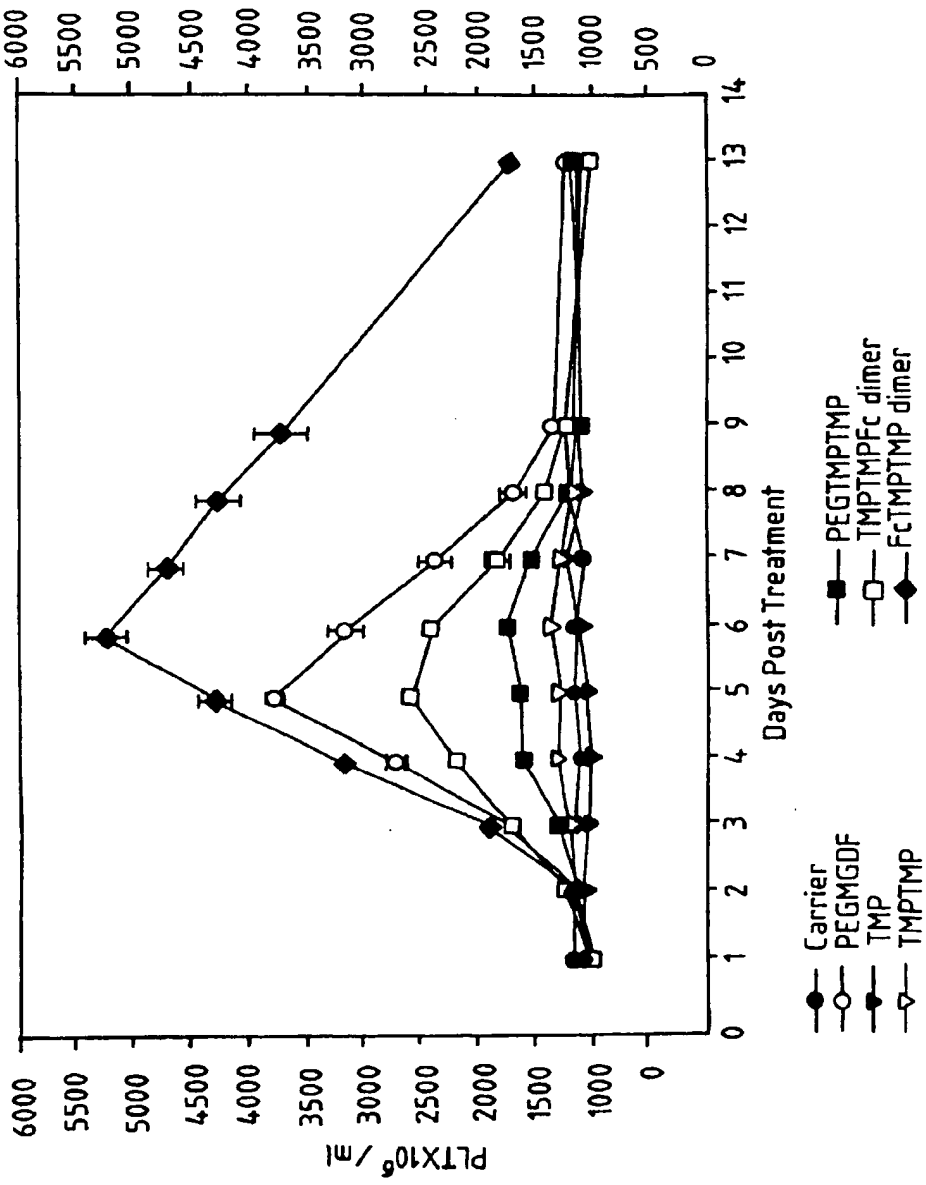

PLTX10⁶/ml
Days Post Treatment
Carrier
PEGMGDF
TMP
TMPTMP
PEGTMPTMP
TMPTMPFc dimer
FcTMPTMP dimer

FIG. 5

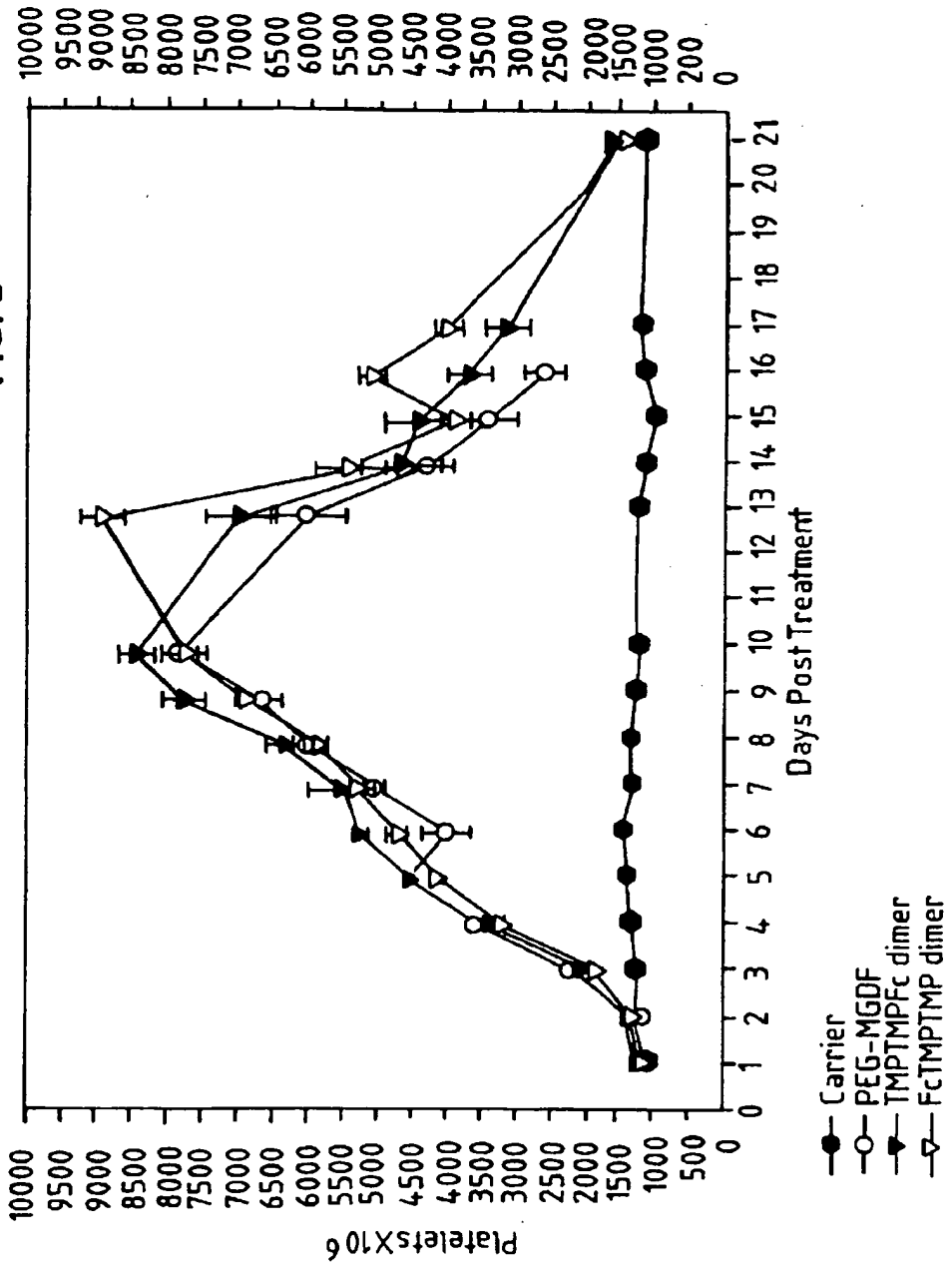

Platelets X10⁶
Days Post Treatment
Carrier
PEG-MGDF
TMPTMPFc dimer
FcTMPTMP dimer

FIG. 6A

FIG. 6B

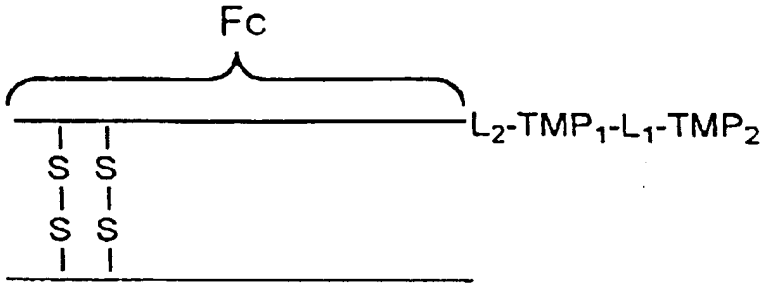
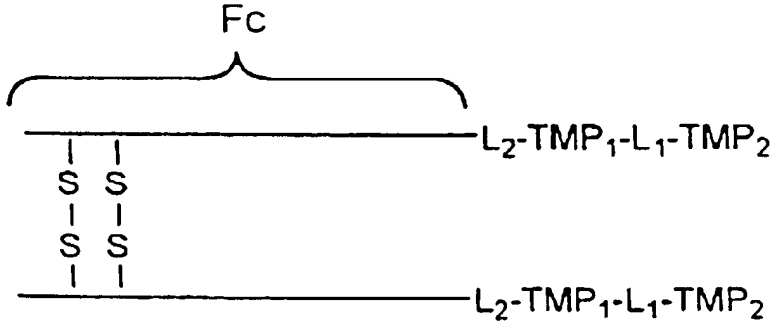


FIG. 6C



THROMBOPOIETIC COMPOUNDS

This application claims priority of U.S. Provisional Application Ser. No. 60/105,348 filed Oct. 23, 1998.

FIELD OF THE INVENTION

Generally, the invention relates to the field of compounds, especially peptides and polypeptides, that have thrombopoietic activity. The compounds of the invention may be used to increase production of platelets or platelet precursors (e.g., megakaryocytes) in a mammal.

BACKGROUND OF THE INVENTION

This invention relates to compounds, especially peptides, that have the ability to stimulate in vitro and in vivo production of platelets and their precursor cells such as megakaryocytes. Before discussing the nature of the inventive compounds, the following is provided as a background regarding two proteins that have thrombopoietic activity: thrombopoietin (TPO) and megakaryocyte growth and development factor (MGDF).

The cloning of endogenous thrombopoietin (TPO) (Lok et al., Nature 369:568–571 (1994); Bartley et al., Cell 77:1117–1124 (1994); Kuter et al., Proc. Natl. Acad. Sci. USA 91:11104–11108 (1994); de Sauvage et al., Nature 369:533–538 (1994); Kato et al., Journal of Biochemistry 119:229–236 (1995), Chang et al., Journal of Biological Chemistry 270:511–514 (1995)) has rapidly increased our understanding of megakaryopoiesis (megakaryocyte production) and thrombopoiesis (platelet production).

Endogenous human TPO, a 60 to 70 kDa glycosylated protein primarily produced in the liver and kidney, consists of 332 amino acids (Bartley et al., Cell 77:1117–1124 (1994); Chang et al., Journal of Biological Chemistry 270:511–514 (1995)). The protein is highly conserved between different species, and has 23% homology with human erythropoietin (Gurney et al., Blood 85:981–988 (1995)) in the amino terminus (amino acids 1 to 172) (Bartley et al., Cell 77:1117–1124 (1994)). Endogenous TPO has been shown to possess all of the characteristics of the key biological regulator of thrombopoiesis. Its in vitro actions include specific induction of megakaryocyte colonies from both purified murine hematopoietic stem cells (Zeigler et al., Blood 84:4045–4052 (1994)) and human $CD34^+$ cells (Lok et al., Nature 369:568–571 (1994); Rasko et al., Stem Cells 15:33–42 (1997)), the generation of megakaryocytes with increased ploidy (Broudy et al., Blood 85:402–413 (1995)), and the induction of terminal megakaryocyte maturation and platelet production (Zeigler et al., Blood 84:4045–4052 (1994); Choi et al., Blood 85:402–413 (1995)). Conversely, synthetic antisense oligodeoxynucleotides to the TPO receptor (c-MP1) significantly inhibit the colony-forming ability of megakaryocyte progenitors (Methia et al., Blood 82:1395–1401 (1993)). Moreover, c-MP1 knock-out mice are severely thrombocytopenic and deficient in megakaryocytes (Alexander et al., Blood 87:2162–2170 (1996)).

Recombinant human MGDF (rHuMGDF, Amgen Inc., Thousand Oaks, Calif.) is another thrombopoietic polypeptide related to TPO. It is produced using *E. coli* transformed with a plasmid containing cDNA encoding a truncated protein encompassing the amino-terminal receptor-binding domain of human TPO (Ulich et al., Blood 86:971–976 (1995)). The polypeptide is extracted, refolded, and purified, and a poly[ethylene glycol] (PEG) moiety is covalently attached to the amino terminus. The resulting molecule is referred to herein as PEG-rHuMGDF or MGDF for short.

Various studies using animal models (Ulich, T. R. et al., Blood 86:971–976 (1995); Hokom, M. M. et al., Blood 86:4486–4492 (1995)) have clearly demonstrated the therapeutic efficacies of TPO and MGDF in bone marrow transplantation and in the treatment of thrombocytopenia, a condition that often results from chemotherapy or radiation therapy. Preliminary data in humans have confirmed the utility of MGDF in elevating platelet counts in various settings. (Basser et al., Lancet 348:1279–81 (1996); Kato et al., Journal of Biochemistry 119:229–236 (1995); Ulich et al., Blood 86:971–976 (1995)). MGDF might be used to enhance the platelet donation process, since administration of MGDF increases circulating platelet counts to about three-fold the original value in healthy platelet donors.

TPO and MGDF exert their action through binding to the c-MP1 receptor which is expressed primarily on the surface of certain hematopoietic cells, such as megakaryocytes, platelets, $CD34^+$ cells and primitive progenitor cells (Debili, N. et al., Blood 85:391–401 (1995); de Sauvage, F. J. et al, Nature 369:533–538 (1994); Bartley, T. D., et al., Cell 77:1117–1124 (1994); Lok, S. et al., Nature 369: 565-S (1994)). Like most receptors for interleukins and protein hormones, c-MP1 belongs to the class I cytokine receptor superfamily (Vigon, I. et al., Proc. Natl. Acad. Sci. USA 89:5640–5644 (1992)). Activation of this class of receptors involves ligand-binding induced receptor homodimerization which in turn triggers the cascade of signal transducing events.

In general, the interaction of a protein ligand with its receptor often takes place at a relatively large interface. However, as demonstrated in the case of human growth hormone bound to its receptor, only a few key residues at the interface actually contribute to most of the binding energy (Clackson, T. et al., Science 267:383–386 (1995)). This and the fact that the bulk of the remaining protein ligand serves only to display the binding epitopes in the right topology makes it possible to find active ligands of much smaller size.

In an effort toward this, the phage peptide library display system has emerged as a powerful technique in identifying small peptide mimetics of large protein ligands (Scott, J. K. et al., Science 249:386 (1990); Devlin, J. J. et al., Science 249:404 (1990)). By using this technique, small peptide molecules that act as agonists of the c-MP1 receptor were discovered (Cwirla, S. E. et al., Science 276:1696–1699 (1997)).

In such a study, random small peptide sequences displayed as fusions to the coat proteins of filamentous phage were affinity-eluted against the antibody-immobilized extracellular domain of c-MP1 and the retained phages were enriched for a second round of affinity purification. This binding selection and repropagation process was repeated many times to enrich the pool of tighter binders. As a result, two families of c-MP1-binding peptides, unrelated to each other in their sequences, were first identified. Mutagenesis libraries were then created to further optimize the best binders, which finally led to the isolation of a very active peptide with an IC_{50}=2 nM and an EC_{50}=400 nM (Cwirla, S. E. et al., Science 276:1696–1699 (1997)). This 14-residue peptide, designated as a TMP (for TPO Mimetic Peptide), has no apparent sequence homology to TPO or MGDF. The structure of this TMP compound is as follows:

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala SEQ ID NO: 1

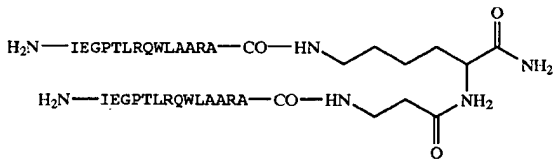
or

IEGPTLRQWLAARA using single letter amino acid abbreviations.

Previously, in a similar study on EPO mimetic peptides, an EPO mimetic peptide (EMP) was discovered using the same technique (Wrighton, N. C. et al., Science 273:458–463 (1996)), and was found to act as a dimer in binding to the EPO receptor (EPOR). The $(ligand)_2$/$(receptor)_2$ complex thus formed had a C2 symmetry according to X-ray crystallographic data (Livnah, O. et al., Science 273:464–471 (1996)). Based on this structural information, a covalently linked dimer of EMP in which the C-termini of two EMP monomers were crosslinked with a flexible spacer was designed and found to have greatly enhanced binding as well as in vitro/in vivo bioactivity (Wrighton, N. C., et al., Nature Biotechnology 15: 1261–1265 (1997)).

A similar C-terminal dimerization strategy was applied to the TPO mimetic peptide (TMP) (Cwirla, S. E. et al., Science 276:1696–1699 (1997)). It was found that a C-terminally linked dimer (C—C link) of TMP had an improved binding affinity of 0.5 nM and a remarkably increased in vitro activity (EC_{50}=0.1 nM) in cell proliferation assays (Cwirla, S. E. et al., Science 276:1696–1699 (1997)). The structure of this TMP C—C dimer is shown below:

(SEQ ID NO: 2)



In another aspect of the present invention, the tandem dimers may be further attached to one or more moieties that are derived from immunoglobulin proteins, referred to generally as the Fc region of such immunoglobulins. The resulting compounds are referred to as Fc fusions of TMP tandem dimers.

The following is a brief background section relating to the Fc regions of antibodies that are useful in connection with some of the present compounds.

Antibodies comprise two functionally independent parts, a variable domain known as "Fab", which binds antigen, and a constant domain, known as "Fc" which provides the link to effector functions such as complement fixation or phagocytosis. The Fc portion of an immunoglobulin has a long plasma half-life, whereas the Fab is short-lived. (Capon, et al., Nature 337:525–531 (1989)).

Therapeutic protein products have been constructed using the Fc domain to attempt to provide longer half-life or to incorporate functions such as Fc receptor binding, protein A binding, complement fixation, and placental transfer which all reside in the Fc region of immunoglobulins (Capon, et al., Nature 337:525–531 (1989)). For example, the Fc region of an IgG1 antibody has been fused to CD30-L, a molecule which binds CD30 receptors expressed on Hodgkin's Disease tumor cells, anaplastic lymphoma cells, T-cell leukemia cells and other malignant cell types. See, U.S. Pat. No. 5,480,981. IL-10, an anti-inflammatory and antirejection agent has been fused to murine Fcγ2a in order to increase the cytokine's short circulating half-life (Zheng, X. et al., The Journal of Immunology, 154; 5590–5600 (1995)). Studies have also evaluated the use of tumor necrosis factor receptor linked with the Fc protein of human IgG1 to treat patients with septic shock (Fisher, C. et al., N. Engl. J. Med., 334: 1697–1702 (1996); Van Zee, K. et al., The Journal of Immunology, 156: 2221–2230 (1996)). Fc has also been fused with CD4 receptor to produce a therapeutic protein for treatment of AIDS. See, Capon et al., Nature, 337:525–531 (1989). In addition, interleukin 2 has been fused to the Fc portion of IgG1 or IgG3 to overcome the short half life of interleukin 2 and its systemic toxicity. See, Harvill et al., Immunotechnology, 1: 95–105 (1995).

In spite of the availability of TPO and MGDF, there remains a need to provide additional compounds that have a biological activity of stimulating the production of platelets (thrombopoietic activity) and/or platelet precursor cells, especially megakaryocytes (megakaryopoietic activity). The present invention provides new compounds having such activity(ies), and related aspects.

SUMMARY OF THE INVENTION

The present invention provides a group of compounds that are capable of binding to and triggering a transmembrane signal through, i.e., activating, the c-MP1 receptor, which is the same receptor that mediates the activity of endogenous thrombopoietin (TPO). Thus, the inventive compounds have thrombopoietic activity, i.e., the ability to stimulate, in vivo and in vitro, the production of platelets, and/or megakaryocytopoietic activity, i.e., the ability to stimulate, in vivo and in vitro, the production of platelet precursors.

In a first preferred embodiment, the inventive compounds comprise the following general structure:

$$TMP_1\text{-}(L_1)_n\text{-}TMP_2$$

wherein TMP_1 and TMP_2 are each independently selected from the group of compounds comprising the core structure: $X_2\text{-}X_3\text{-}X_4\text{-}X_5\text{-}X_6\text{-}X_7\text{-}X_8\text{-}X_9\text{-}X_{10}$,
wherein,

X2 is selected from the group consisting of Glu, Asp, Lys, and Val;

X3 is selected from the group consisting of Gly and Ala;

X4 is Pro;

X5 is selected from the group consisting of Thr and Ser;

X6 is selected from the group consisting of Leu, Ile, Val, Ala, and Phe;

X7 is selected from the group consisting of Arg and Lys;

X8 is selected from the group consisting of Gln, Asn, and Glu;

X9 is selected from the group consisting of Trp, Tyr, Cys, Ala, and Phe;

X10 is selected from the group consisting of Leu, Ile, Val, Ala, Phe, Met, and Lys;

L1 is a linker as described herein; and

n is 0 or 1;

and physiologically acceptable salts thereof.

In one embodiment, L_1 comprises $(Gly)_n$, wherein n is 1 through 20, and when n is greater than 1, up to half of the Gly residues may be substituted by another amino acid selected from the remaining 19 natural amino acids or a stereoisomer thereof.

In addition to the core structure X_2–X_{10} set forth above for TMP_1, and TMP_2, other related structures are also possible wherein one or more of the following is added to the TMP_1 and/or TMP_2 core structure: X_1 is attached to the N-terminus and/or X_{11}, X_{12}, X_{13}, and/or X_{14} are attached to the C-terminus, wherein X_1, X_{12}, X_{13}, and X_{14} are as follows:

X_1 is selected from the group consisting of Ile, Ala, Val, Leu, Ser, and Arg;

X_{11} is selected from the group consisting of Ala, Ile, Val, Leu, Phe, Ser, Thr, Lys, His, and Glu;

X_{12} is selected from the group consisting of Ala, Ile, Val, Leu, Phe, Gly, Ser, and Gln;

X_{13} is selected from the group consisting of Arg, Lys, Thr, Val, Asn, Gln, and Gly; and

X_{14} is selected from the group consisting of Ala, Ile, Val, Leu, Phe, Thr, Arg, Glu, and Gly.

In a second preferred embodiment, the inventive compounds have the general formula:

$$(Fc)_m\text{-}(L_2)_q\text{-}TMP_1\text{-}(L_1)_n\text{-}TMP_2\text{-}(L_3)_r\text{-}(Fc)_p$$

wherein TMP_1, TMP_2 and n are each as described above; L_1, L_2 and L_3 are linker groups which are each independently selected from the linker groups described herein; Fc is an Fc region of an immunoglobulin (as defined herein below); m, p, q and r are each independently selected from the group consisting of 0 and 1, wherein at least one of m or p is 1, and further wherein if m is 0 then q is 0, and if p is 0, then r is 0; and physiologically acceptable salts thereof. In one embodiment, L_1, L_2, and L_3 independently comprise $(Gly)_n$, wherein n is 1 through 20, and when n is greater than 1, up to half of the Gly residues may be substituted by another amino acid selected from the remaining 19 natural amino acids or a stereoisomer thereof.

Derivatives of the above compounds (described below) are also encompassed by this invention.

The compounds of this invention are preferably peptides, and they may be prepared by standard synthetic methods or any other methods of preparing peptides. The compounds of this invention that encompass non-peptide portions may be synthesized by standard organic chemistry reactions, in addition to standard peptide chemistry reactions when applicable.

The compounds of this invention may be used for therapeutic or prophylactic purposes by incorporating them with appropriate pharmaceutical carrier materials and administering an effective amount to a subject, such as a human (or other mammal). Other related aspects are also included in the instant invention.

BRIEF DESCRIPTION OF THE FIGURES

Numerous other aspects and advantages of the present invention will therefore be apparent upon consideration of the following detailed description thereof, reference being made to the drawings wherein:

FIG. 1 shows exemplary Fc polynucleotide and protein sequences (SEQ ID NO: 3 is the coding strand reading 5'-3', SEQ ID NO: 4 is the complementary strand reading 3'-5'; and SEQ ID NO: 5 is the encoded amino acids sequence) of human IgG1 that may be used in the Fc fusion compounds of this invention.

FIG. 2 shows a synthetic scheme for the preparation of pegylated peptide 19 (SEQ ID NO: 17).

FIG. 3 shows a synthetic scheme for the preparation of pegylated peptide 20 (SEQ ID NO: 18).

FIG. 4 shows the number of platelets generated in vivo in normal female BDF1 mice treated with one 100 μg/kg bolus injection of various compounds, as follows: PEG-MGDF means 20 kD average molecular weight PEG attached to the N-terminal amino group by reductive amination of amino acids 1–163 of native human TPO, which is expressed in *E. coli* (so that it is not glycosylated) (See WO 95/26746 entitled "Compositions and Methods for Stimulating Megakaryocyte Growth and Differentiation"); TMP means the compound of SEQ ID NO: 1; TMP-TMP means the compound of SEQ ID NO: 21; PEG-TMP-TMP means the compound of SEQ ID NO: 18, wherein the PEG group is a 5 kD average molecular weight PEG attached as shown in FIG. 3; TMP-TMP-Fc is defined herein below and Fc-TMP-TMP is the same as TMP-TMP-Fc except that the Fc group is attached at the N-terminal end rather than the C-terminal end of the TMP-TMP peptide.

FIG. 5 shows the number of platelets generated in vivo in normal BDF1 mice treated with various compounds delivered via implanted osmotic pumps over a 7-day period. The compounds are defined in the same manner as set forth above for FIG. 4.

FIGS. 6A, 6B, and 6C show schematic diagrams of preferred compounds of the present invention. FIG. 6A shows an Fc fusion compound wherein the Fc moiety is fused at the N-terminus of the TMP dimer, and wherein the Fc portion is a monomeric (single chain) form. FIG. 6B shows an Fc fusion compound wherein the Fc region is fused at the N-terminus of the TMP dimer, and wherein the Fc portion is dimeric, and one Fc monomer is attached to a TMP dimer. FIG. 6C shows an Fc fusion compound wherein the Fc moiety is fused at the N-terminus of the TMP dimer, and wherein the Fc portion is dimeric and each Fc monomer is attached to a TMP dimer.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In an effort to seek small structures as lead compounds in the development of therapeutic agents with more desirable properties, a different type of dimer of TMP and related structures were designed in which the C-terminus of one TMP peptide was linked to the N-terminus of a second TMP peptide, either directly or via a linker and the effects of this dimerization strategy on the bioactivity of the resulting dimeric molecules were then investigated. In some cases, these so-called tandem dimers (C–N link) were designed to have linkers between the two monomers, the linkers being preferably composed of natural amino acids, therefore rendering their synthesis accessible to recombinant technologies.

The present invention is based on the discovery of a group of compounds that have thrombopoietic activity and which are thought to exert their activity by binding to the endogenous TPO receptor, c-MP1.

Compounds and Derivatives

In a first preferred embodiment, the inventive compounds comprise the following general structure:

$$TMP_1\text{-}(L_1)_n\text{-}TMP_2$$

wherein TMP_1, and TMP_2 are each independently selected from the group of compounds comprising the core structure: $X_2\text{-}X_3\text{-}X_4\text{-}X_5\text{-}X_6\text{-}X_7\text{-}X_8\text{-}X_9\text{-}X_{10}$,

wherein,

X_2 is selected from the group consisting of Glu, Asp, Lys, and Val;

X_3 is selected from the group consisting of Gly and Ala;

X_4 is Pro;

X_5 is selected from the group consisting of Thr and Ser;

X_6 is selected from the group consisting of Leu, Ile, Val, Ala, and Phe;

X_7 is selected from the group consisting of Arg and Lys;

X_8 is selected from the group consisting of Gln, Asn, and Glu;

X_9 is selected from the group consisting of Trp, Tyr, and Phe;

X_{10} is selected from the group consisting of Leu, Ile, Val, Ala, Phe, Met, and Lys;

L_1 is a linker as described herein; and

n is 0 or 1;

and physiologically acceptable salts thereof.

In one embodiment, L_1, comprises $(Gly)_n$, wherein n is 1 through 20, and when n is greater than 1, up to half of the Gly residues may be substituted by another amino acid selected from the remaining 19 natural amino acids or a stereoisomer thereof.

In addition to the core structure X_2–X_{10} set forth above for TMP_1, and TMP_2, other related structures are also possible wherein one or more of the following is added to the TMP_1 and/or TMP_2 core structure: X_1 is attached to the N-terminus and/or X_{11}, X_{12}, X_{13}, and/or X_{14} are attached to the C-terminus, wherein X_1, X_{11}, X_{12}, X_{13}, and X_{14} are as follows:

X_1 is selected from the group consisting of Ile, Ala, Val, Leu, Ser, and Arg;

X_{11}, is selected from the group consisting of Ala, Ile, Val, Leu, Phe, Ser, Thr, Lys, His, and Glu;

X_{12} is selected from the group consisting of Ala, Ile, Val, Leu, Phe, Gly, Ser, and Gln;

X_{13} is selected from the group consisting of Arg, Lys, Thr, Val, Asn, Gln, and Gly; and

X_{14} is selected from the group consisting of Ala, Ile, Val, Leu, Phe, Thr, Arg, Glu, and Gly.

The term "TMP" is used to mean a moiety made up of, i.e., comprising, at least 9 subunits (X_2–X_{10}), wherein X_2–X_{10} comprise the core structure. The X_2–X_{14} subunits are preferably amino acids independently selected from among the 20 naturally-occurring amino acids, however, the invention embraces compounds where X_2–X_{14} are independently selected from the group of atypical, non-naturally occurring amino acids well known in the art. Specific preferred amino acids are identified for each position. For example, X_2 may be Glu, Asp, Lys, or Val. Both three-letter and single letter abbreviations for amino acids are used herein; in each case, the abbreviations are the standard ones used for the 20 naturally-occurring amino acids or well-known variations thereof. These amino acids may have either L or D stereochemistry (except for Gly, which is neither L nor D), and the TMPs may comprise a combination of stereochemistries. However, the L stereochemistry is preferred for all of the amino acids in the TMP chain. The invention also provides reverse TMP molecules wherein the amino terminal to carboxy terminal sequence of the amino acids is reversed. For example, the reverse of a molecule having the normal sequence X_1-X_2-X_3 would be X_3-X_2-X_1. The invention also provides retro-reverse TMP molecules wherein, like a reverse TMP, the amino terminal to carboxy terminal sequence of amino acids is reversed and residues that are normally "L" enatiomers in TMP are altered to the "D" stereoisomer form.

Additionally, physiologically acceptable salts of the TMPs are also encompassed. "Physiologically acceptable salts" means any salts that are known or later discovered to be pharmaceutically acceptable. Some specific preferred examples are: acetate, trifluoroacetate, hydrochloride, hydrobromide, sulfate, citrate, tartrate, glycolate, oxalate.

It is also contemplated that "derivatives" of the TMPs may be substituted for the above-described TMPs. Such derivative TMPs include moieties wherein one or more of the following modifications have been made:

one or more of the peptidyl [—C(O)NR—] linkages (bonds) have been replaced by a non-peptidyl linkage such as a —CH_2-carbamate linkage [—CH_2—OC(O)NR—]; a phosphonate linkage; a —CH_2-sulfonamide [—CH_2—$S(O)_2$NR—] linkage; a urea [—NHC(O)NH—] linkage; a —CH_2-secondary amine linkage; or an alkylated peptidyl linkage [—C(O)NR^6— where R^6 is lower alkyl];

peptides wherein the N-terminus is derivatized to a —NRR^1 group; to a —NRC(O)R group; to a —NRC(O)OR group; to a —$NRS(O)_2$R group; to a —NHC(O)NHR group, where R and R^1 are hydrogen or lower alkyl, with the proviso that R and R^1 are not both hydrogen; to a succinimide group; to a benzyloxycarbonyl-NH— (CBZ—NH—) group; or to a benzyloxycarbonyl-NH— group having from 1 to 3 substituents on the phenyl ring selected from the group consisting of lower alkyl, lower alkoxy, chloro, and bromo; and

peptides wherein the free C terminus is derivatized to —$C(O)R^2$ where R^2 is selected from the group consisting of lower alkoxy and —NR^3R^4 where R^3 and R^4 are independently selected from the group consisting of hydrogen and lower alkyl. By "lower" is meant a group having from 1 to 6 carbon atoms.

Additionally, modifications of individual amino acids may be introduced into the TMP molecule by reacting targeted amino acid residues of the peptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues. The following are exemplary:

Lysinyl and amino terminal residues may be reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing alpha-amino-containing residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea, 2,4 pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues may be modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pKa of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine guanidino group.

The specific modification of tyrosyl residues per se has been studied extensively, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidizole and tetranitromethane may be used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively.

Carboxyl side groups (aspartyl or glutamyl) may be selectively modified by reaction with carbodiimides (R^1—N=C=N—R^1) such as 1-cyclohexyl-3-(2-morpholinyl-(4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues may be converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues may be deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Derivatization with bifunctional agents is useful for cross-linking the peptides or their functional derivatives to a

water-insoluble support matrix or to other macromolecular carriers. Commonly used cross-linking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis (succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Pat. Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 may be employed for protein immobilization.

Other possible modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, oxidation of the sulfur atom in Cys, methylation of the alpha-amino groups of lysine, arginine, and histidine side chains (Creighton, T. E., Proteins: Structure and Molecule Properties, W. H. Freeman & Co., San Francisco, pp. 79–86 (1983)), acetylation of the N-terminal amine, and, in some instances, amidation of the C-terminal carboxyl groups.

Such derivatized moieties preferably improve one or more characteristics including thrombopoietic activity, solubility, absorption, biological half life, and the like of the inventive compounds. Alternatively, derivatized moieties result in compounds that have the same, or essentially the same, characteristics and/or properties of the compound that is not derivatized. The moieties may alternatively eliminate or attenuate any undesirable side effect of the compounds and the like.

In addition to the core structure set forth above, X_2–X_{10}, other structures that are specifically contemplated are those in which one or more additional X groups are attached to the core structure. Thus, X_1, and/or X_{11}, X_{12}, X_{13}, and X_{14} may be attached to the core structure. Some exemplary additional structures are the following:

X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11};
X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-X_{12};
X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-X_{12}-X_{13};
X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-X_{12}-X_{13}-X_{14};
X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10};
X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11};
X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-X_{12};
X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-X_{12}-X_{13};
X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-X_{12}-X_{13}-X_{14},

wherein X_1 through X_{14} are as described above. Each of TMP_1 and TMP_2 may be the same or different in sequence and/or length. In some preferred embodiments, TMP_1 and TMP_2 are the same.

A particularly preferred TMP is the following:

Ile-Glu-Gly-Pro-Thr-Leu-Arg-Gln-Trp-Leu-Ala-Ala-Arg-Ala (SEQ ID NO: 1).

As used herein "comprising" means, inter alia, that a compound may include additional amino acids on either or both of the - or C- termini of the given sequence. However, as long as a structure such as X_2 to X_{10}, X_1 to X_{14}, or one of the other exemplary structures is present, the remaining chemical structure is relatively less important. Of course, any structure outside of the core X_2 to X_{10} structure, or the X_1 to X_{14}, structure, should not significantly interfere with thrombopoietic activity of the compound. For example, an N-terminal Met residue is envisioned as falling within this invention. Additionally, although many of the preferred compounds of the invention are tandem dimers in that they possess two TMP moieties, other compounds of this invention are tandem multimers of the TMPs, i.e., compounds of the following exemplary structures:

TMP_1-L-TMP_2-L-TMP_3;
TMP_1-L-TMP_2-L-TMP_3-L-TMP_4;
TMP_1-L-TMP_2-L-TMP_3-L-TMP_4-L-TMP_5;

wherein TMP_1, TMP_2, TMP_3, TMP_4, and TMP_5 can have the same or different structures, and wherein each TMP and L is defined as set forth herein, and the linkers are each optional. Preferably, the compounds of this invention will have from 2–5 TMP moieties, particularly preferably 2–3, and most preferably 2. The compounds of the first embodiment of this invention will preferably have less than about 60, more preferably less than about 40 amino acids in total (i.e., they will be peptides).

As noted above, the compounds of the first embodiment of this invention are preferably TMP dimers which are either bonded directly or are linked by a linker group. The monomeric TMP moieties are shown in the conventional orientation from N to C terminus reading from left to right. Accordingly, it can be seen that the inventive compounds are all oriented so that the C terminus of TMP_1 is attached either directly or through a linker to the N-terminus of TMP_2. This orientation is referred to as a tandem orientation, and the inventive compounds may be generally referred to as "tandem dimers". These compounds are referred to as dimers even if TMP_1 and TMP_2 are structurally distinct. That is, both homodimers and heterodimers are envisioned.

The "linker" group ("L_1") is optional. When it is present, it is not critical what its chemical structure is, since it serves primarily as a spacer. The linker should be chosen so as not to interfere with the biological activity of the final compound and also so that immunogenicity of the final compound is not significantly increased. The linker is preferably made up of amino acids linked together by peptide bonds. Thus, in preferred embodiments, the linker comprises Y_n, wherein Y is a naturally occurring amino acid or a steroisomer thereof and "n" is any one of 1 through 20. The linker is therefore made up of from 1 to 20 amino acids linked by peptide bonds, wherein the amino acids are selected from the 20 naturally-occurring amino acids. In a more preferred embodiment, the 1 to 20 amino acids are selected from Gly, Ala, Pro, Asn, Gln, Cys, Lys. Even more preferably, the linker is made up of a majority of amino acids that are sterically un-hindered, such as Gly, Gly-Gly [$(Gly)_2$], Gly-Gly-Gly [$(Gly)_3$] . . . $(Gly)_{20}$, Ala, Gly-Ala, Ala-Gly, Ala-Ala, etc. Other specific examples of linkers are:

$(Gly)_3Lys(Gly)_4$ (SEQ ID NO: 6);
$(Gly)_3AsnGlySer(Gly)_2$ (SEQ ID NO: 7)
(this structure provides a site for glycosylation, when it is produced recombinantly in a mammalian cell system that is capable of glycosylating such sites);
$(Gly)_3Cys(Gly)_4$ (SEQ ID NO: 8); and
GlyProAsnGly (SEQ ID NO: 9).

To explain the above nomenclature, for example, $(Gly)_3Lys(Gly)_4$ means Gly-Gly-Gly-Lys-Gly-Gly-Gly-Gly. Combinations of Gly and Ala are also preferred.

Non-peptide linkers are also possible. For example, alkyl linkers such as —HN—$(CH_2)_s$—CO—, wherein s=2–20 could be used. These alkyl linkers may further be substituted by any non-sterically hindering group such as lower alkyl (e.g., C_1–C_6), lower acyl, halogen (e.g., Cl, Br), CN, NH_2, phenyl, etc.

Another type of non-peptide linker is a polyethylene glycol group, such as:

wherein n is such that the overall molecular weight of the linker ranges from approximately 101 to 5000, preferably 101 to 500.

In general, it has been discovered that a linker of a length of about 0–14 sub-units (e.g., amino acids) is preferred for the thrombopoietic compounds of the first embodiment of this invention.

The peptide linkers may be altered to form derivatives in the same manner as described above for the TMPs.

The compounds of this first group may further be linear or cyclic. By "cyclic" is meant that at least two separated, i.e., non-contiguous, portions of the molecule are linked to each other. For example, the amino and carboxy terminus of the ends of the molecule could be covalently linked to form a cyclic molecule. Alternatively, the molecule could contain two or more Cys residues (e.g., in the linker), which could cyclize via disulfide bond formation. It is further contemplated that more than one tandem peptide dimer can link to form a dimer of dimers. Thus, for example, a tandem dimer containing a Cys residue can form an intermolecular disulfide bond with a Cys of another such dimer. See, for example, the compound of SEQ ID NO: 20, below.

The compounds of the invention may also be covalently or noncovalently associated with a carrier molecule, such as a linear polymer (e.g., polyethylene glycol, polylysine, dextran, etc.), a branched-chain polymer (see, for example, U.S. Pat. No. 4,289,872 to Denkenwalter et al., issued Sep. 15, 1981; 5,229,490 to Tam, issued Jul. 20, 1993; WO 93/21259 by Frechet et al., published 28 October 1993); a lipid; a cholesterol group (such as a steroid); or a carbohydrate or oligosaccharide. Other possible carriers include one or more water soluble polymer attachments such as polyoxyethylene glycol, or polypropylene glycol as described U.S. Pat. Nos.: 4,640,835, 4,496,689, 4,301,144, 4,670,417, 4,791,192 and 4,179,337. Still other useful polymers known in the art include monomethoxy-polyethylene glycol, dextran, cellulose, or other carbohydrate based polymers, poly-(N-vinyl pyrrolidone)-polyethylene glycol, propylene glycol homopolymers, a polypropylene oxide/ethylene oxide co-polymer, polyoxyethylated polyols (e.g., glycerol) and polyvinyl alcohol, as well as mixtures of these polymers.

A preferred such carrier is polyethylene glycol (PEG). The PEG group may be of any convenient molecular weight and may be straight chain or branched. The average molecular weight of the PEG will preferably range from about 2 kDa to about 100 kDa, more preferably from about 5 kDa to about 50 kDa, most preferably from about 5 kDa to about 10 kDa.

The PEG groups will generally be attached to the compounds of the invention via acylation, reductive alkylation, Michael addition, thiol alkylation or other chemoselective conjugation/ligation methods through a reactive group on the PEG moiety (e.g., an aldehyde, amino, ester, thiol, α-haloacetyl, maleimido or hydrazino group) to a reactive group on the target compound (e.g., an aldehyde, amino, ester, thiol, α-haloacetyl, maleimido or hydrazino group).

Carbohydrate (oligosaccharide) groups may conveniently be attached to sites that are known to be glycosylation sites in proteins. Generally, O-linked oligosaccharides are attached to serine (Ser) or threonine (Thr) residues while N-linked oligosaccharides are attached to asparagine (Asn) residues when they are part of the sequence Asn-X-Ser/Thr, where X can be any amino acid except proline. X is preferably one of the 19 naturally occurring amino acids not counting proline. The structures of N-linked and O-linked oligosaccharides and the sugar residues found in each type are different. One type of sugar that is commonly found on both is N-acetylneuraminic acid (referred to as sialic acid). Sialic acid is usually the terminal residue of both N-linked and O-linked oligosaccharides and, by virtue of its negative charge, may confer acidic properties to the glycosylated compound. Such site(s) may be incorporated in the linker of the compounds of this invention and are preferably glycosylated by a cell during recombinant production of the polypeptide compounds (e.g., in mammalian cells such as CHO, BHK, COS). However, such sites may further be glycosylated by synthetic or semi-synthetic procedures known in the art.

Some exemplary peptides of this invention are shown below. Single letter amino acid abbreviations are used, and the linker is shown separated by dashes for clarity. Additional abbreviations: BrAc means bromoacetyl ($BrCH_2C(O)$) and PEG is polyethylene glycol.

(SEQ ID NO: 10)

IEGPTLRQWLAARA-GPNG-IEGPTLRQWLAARA

(SEQ ID NO: 11)

IEGPTLRQCLAARA-GGGGGGGG-IEGPTLRQCLAARA (cyclic)

(SEQ ID NO: 12)

IEGPTLRQCLAARA-GGGGGGGG-IEGPTLRQCLAARA (linear)

(SEQ ID NO: 13)

IEGPTLRQALAARA-GGGGGGGG-IEGPTLRQALAARA

(SEQ ID NO: 14)

IEGPTLRQWLAARA-GGGKGGGG-IEGPTLRQWLAARA

(SEQ ID NO: 15)

IEGPTLRQWLAARA-GGGK(BrAc)GGGG-IEGPTLRQWLAARA

(SEQ ID NO: 16)

IEGPTLRQALAARA-GGGCGGGG-IEGPTLRQWLAARA

(SEQ ID NO: 17)

IEGPTLRQWLAARA-GGGK(PEG)GGGG-IEGPTLRQWLAARA

(SEQ ID NO: 18)

IEGPTLRQWLAARA-GGGC(PEG)GGGG-IEGPTLRQWLAARA

(SEQ ID NO: 19)

IEGPTLRQWLAARA-GGGNGSGG-IEGPTLRQWLAARA

(SEQ ID NO: 20)

IEGPTLRQWLAARA-GGGCGGGG-IEGPTLRQWLAARA

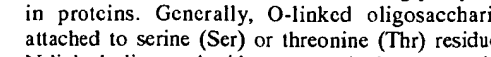
IEGPTLRQWLAARA-GGGCGGGG-IEGPTLRQWLAARA

(SEQ ID NO: 21)

IEGPTLRQWLAARA-GGGGGGGG-IEGPTLRQWLAARA

In each of the above compounds, an N-terminal Met (or M residue, using the one-letter code) is contemplated as well. Multimers (e.g., tandem and non-tandem, covalently bonded and non-covalently bonded) of the above compounds are also contemplated.

In a second embodiment of this invention, the compounds described above may further be fused to one or more Fc groups, either directly or through linker groups. In general, the formula of this second group of compounds is:



wherein TMP_1, TMP_2 and n are each as described above; L_1, L_2 and L_3 are linker groups which are each independently

selected from the linker groups described above; Fc is an Fc region of an immunoglobulin; m, p, q and r are each independently selected from the group consisting of 0 and 1, wherein at least one of m or p is 1, and further wherein if m is 0 then q is 0, and if p is 0, then r is 0; and physiologically acceptable salts thereof.

Accordingly, the compounds of this second group have structures as defined for the first group of compounds as described above, but these compounds are further fused to at least one Fc group either directly or through one or more linker groups.

The Fc sequence of the above compounds may be selected from the human immunoglobulin IgG-1 heavy chain, see Ellison, J. W. et al., Nucleic Acids Res. 10:4071–4079 (1982), or any other Fc sequence known in the art (e.g. other IgG classes including but not limited to IgG-2, IgG-3 and IgG-4, or other immunoglobulins).

It is well known that Fc regions of antibodies are made up of monomeric polypeptide segments that may be linked into dimeric or multimeric forms by disulfide bonds or by non-covalent association. The number of intermolecular disulfide bonds between monomeric subunits of native Fc molecules ranges from 1 to 4 depending on the class (e.g., IgG, IgA, IgE) or subclass (e.g., IgG1, IgG2, IgG3, IgA1, IgGA2) of antibody involved. The term "Fc" as used herein is generic to the monomeric, dimeric, and multimeric forms of Fc molecules. It should be noted that Fc monomers will spontaneously dimerize when the appropriate Cys residues are present unless particular conditions are present that prevent dimerization through disulfide bond formation. Even if the Cys residues that normally form disulfide bonds in the Fc dimer are removed or replaced by other residues, the monomeric chains will generally dimerize through non-covalent interactions. The term "Fc" herein is used to mean any of these forms: the native monomer, the native dimer (disulfide bond linked), modified dimers (disulfide and/or non-covalently linked), and modified monomers (i.e., derivatives).

Variants, analogs or derivatives of the Fc portion may be constructed by, for example, making various substitutions of residues or sequences.

Variant (or analog) polypeptides include insertion variants, wherein one or more amino acid residues supplement an Fc amino acid sequence. Insertions may be located at either or both termini of the protein, or may be positioned within internal regions of the Fc amino acid sequence. Insertional variants with additional residues at either or both termini can include for example, fusion proteins and proteins including amino acid tags or labels. For example, the Fc molecule may optionally contain an N-terminal Met, especially when the molecule is expressed recombinantly in a bacterial cell such as *E. coli*.

In Fc deletion variants, one or more amino acid residues in an Fc polypeptide are removed. Deletions can be effected at one or both termini of the Fc polypeptide, or with removal of one or more residues within the Fc amino acid sequence. Deletion variants, therefore, include all fragments of an Fc polypeptide sequence.

In Fc substitution variants, one or more amino acid residues of an Fc polypeptide are removed and replaced with alternative residues. In one aspect, the substitutions are conservative in nature, however, the invention embraces substitutions that are also non-conservative.

For example, cysteine residues can be deleted or replaced with other amino acids to prevent formation of some or all disulfide crosslinks of the Fc sequences. In particular, the amino acids at positions 7 and 10 of SEQ ID NO:5 are cysteine residues. One may remove each of these cysteine residues or substitute one or more such cysteine residues with other amino acids, such as Ala or Ser. As another example, modifications may also be made to introduce amino acid substitutions to (1) ablate the Fc receptor binding site; (2) ablate the complement (Clq) binding site; and/or to (3) ablate the antibody dependent cell-mediated cytotoxicity (ADCC) site. Such sites are known in the art, and any known substitutions are within the scope of Fc as used herein. For example, see *Molecular Immunology*, Vol. 29, No. 5, 633–639 (1992) with regards to ADCC sites in IgG1.

Likewise, one or more tyrosine residues can be replaced by phenylalanine residues as well. In addition, other variant amino acid insertions, deletions (e.g., from 1–25 amino acids) and/or substitutions are also contemplated and are within the scope of the present invention. Conservative amino acid substitutions will generally be preferred. Furthermore, alterations may be in the form of altered amino acids, such as peptidomimetics or D-amino acids.

Fc sequences of the TMP compound may also be derivatized, i.e., bearing modifications other than insertion, deletion, or substitution of amino acid residues. Preferably, the modifications are covalent in nature, and include for example, chemical bonding with polymers, lipids, other organic, and inorganic moieties. Derivatives of the invention may be prepared to increase circulating half-life, or may be designed to improve targeting capacity for the polypeptide to desired cells, tissues, or organs.

It is also possible to use the salvage receptor binding domain of the intact Fc molecule as the Fc part of the inventive compounds, such as described in WO 96/32478, entitled "Altered Polypeptides with Increased Half-Life". Additional members of the class of molecules designated as Fc herein are those that are described in WO 97/34631, entitled "Immunoglobulin-Like Domains with Increased Half-Lives". Both of the published PCT applications cited in this paragraph are hereby incorporated by reference.

The Fc fusions may be at the N or C terminus of TMP_1 or TMP_2 or at both the N and C termini of the TMPs. It has been surprisingly discovered that peptides in which an Fc moiety is ligated to the N terminus of the TMP group is more bioactive than the other possibilities, so the fusion having an Fc domain at the N terminus of TMP_1 (i.e., r and p are both 0 and m and q are both 1 in general formula) is preferred. When the Fc chain is fused at the N-terminus of the TMP or linker, such fusion will generally occur at the C-terminus of the Fc chain, and vice versa.

Also preferred are compounds that are dimers (e.g., tandem and non-tandem) of the compounds set forth in the general formula as set out above. In such cases, each Fc chain will be linked to a tandem dimer of TMP peptides. A schematic example of such a compound is shown in FIG. 6C. A preferred example of this type of compound is based on FIG. 6C, wherein Fc is a dimer of the compound of SEQ ID NO: 5, each L_2 is $(Gly)_5$, TMP_1 and TMP_2 are each the compound of SEQ ID NO: 1, and each L_1 is $(Gly)_8$. This compound is also referred to herein as "Fc-TMP_1-L-TMP_2". It is also represented as a dimer (through the Fc portion) of SEQ ID NO: 34. The analogous compound wherein the Fc group is attached through a linker to the C-terminus of the TMP_2 groups in FIG. 6C is also contemplated and is referred to herein as TMP_1-L-TMP_2-Fc.

Some specific examples of compounds from the second group are provided as follows:

(SEQ ID NO: 22)
Fc-IEGPTLRQWLAARA-GPNG-IEGPTLRQWLAARA

(SEQ ID NO: 23)
Fc-IEGPTLRQWLAARA-GPNG-IEGPTLRQWLAARA-Fc

(SEQ ID NO: 24)
IEGPTLRQWLAARA-GGGGGGGG-IEGPTLRQWLAARA-Fc

(SEQ ID NO: 25)
Fc-FF-IEGPTLRQWLAARA-GPNG-IEGPTLRQWLAARA

(SEQ ID NO: 26)
Fc-IEGPTLRQWLAARA-GGGGGGGG-IEGPTLRQWLAARA

(SEQ ID NO: 27)
Fc-IEGPTLRQCLAARA-GGGGGGGG-IEGPTLRQCLAARA (cyclic)

(SEQ ID NO: 28)
Fc-IEGPTLRQCLAARA-GGGGGGGG-IEGPTLRQCLAARA (linear)

(SEQ ID NO: 29)
Fc-IEGPTLRQALAARA-GGGGGGGG-IEGPTLRQALAARA

(SEQ ID NO: 30)
Fc-IEGPTLRQWLAARA-GGGKGGGG-IEGPTLRQWLAARA

(SEQ ID NO: 31)
Fc-IEGPTLRQWLAARA-GGGCGGGG-IEGPTLRQWLAARA

(SEQ ID NO: 32)
Fc-IEGPTLRQWLAARA-GGGNGSGG-IEGPTLRQWLAARA

(SEQ ID NO: 33)
Fc-IEGPTLRQWLAARA-GGGCGGGG-IEGPTLRQWLAARA
Fc-IEGPTLRQWLAARA-GGGCGGGG-IEGPTLRQWLAARA

(SEQ ID NO: 34)
Fc-GGGGG-IEGPTLRQWLAARA-GGGGGGGG-IEGPTLRQWLAARA

In each of the above compounds, an additional N-terminal Met (or M residue, using the one-letter code) is contemplated as well. The Met residue may be attached at the N-terminus of the Fc group in those cases wherein there is an Fc group attached to the N-terminus of the TMP. In those cases wherein the Fc group is attached at the C-terminus of the TMP, the Met residues could be attached to the N-terminus of the TMP group.

In each of the above cases Fc is preferably the Fc region of the human immunoglobulin IgG1 heavy chain or a biologically active fragment, derivative, or dimer thereof, see Ellison, J. W. et al., Nucleic Acids Res. 10:4071–4079 (1982). The Fc sequence shown in SEQ ID NO: 5 is the most preferred Fc for the above compounds. Also preferred are the above compounds in which the Fc is a dimeric form of the sequence of SEQ ID NO: 5 and each Fc chain is attached to a TMP tandem dimer.

Additionally, although many of the preferred compounds of the second embodiment include one or more tandem dimers in that they possess two linked TMP moieties, other compounds of this invention include tandem multimers of the TMPs, i.e., compounds of the following exemplary structures:

Fc-TMP_1-L-TMP_2-L-TMP_3;

Fc-TMP_1-L-TMP_2-L-TMP_3-L-TMP_4;

Fc-TMP_1-L-TMP_2-L-TMP_3-L-TMP_4-L-TMP_5;

TMP_1-L-TMP_2-L-TMP_3-L-Fc;

TMP_1-L-TMP_2-L-TMP_3-L-TMP_4-L-Fc;

TMP_1-L-TMP_2-L-TMP_3-L-TMP_4-L-TMP_5-L-Fc;

wherein TMP_1, TMP_2, TMP_3, TMP_4, and TMP_5 can have the same or different structures, and wherein Fc and each TMP and L is defined as set forth above, and the linkers are each optional. In each case above, the Fc group can be monomeric or dimeric, and in cases where the Fc is dimeric, one or more TMP multimer can be attached to each Fc chains. Also contemplated are other examples wherein the TMP dimers or multimers are attached to both the N and C-termini of one or both Fc chains, including the case wherein TMP dimers or multimers are attached to all four termini of two Fc chains.

Preferably, the compounds of this second embodiment of the invention will have from about 200 to 400 amino acids in total (i.e., they will be polypeptides).

Methods of Making

The compounds of this invention may be made in a variety of ways. Since many of the compounds will be peptides, or will include a peptide, methods for synthesizing peptides are of particular relevance here. For example, solid phase synthesis techniques may be used. Suitable techniques are well known in the art, and include those described in Merrifield, in Chem. Polypeptides, pp. 335–61 (Katsoyannis and Panayotis eds. 1973); Merrifield, J. Am. Chem. Soc. 85:2149 (1963); Davis et al., Biochem. Intl. 10:394–414 (1985); Stewart and Young, Solid Phase Peptide Synthesis (1969); U.S. Pat. No. 3,941,763; Finn et al., The Proteins, 3rd ed., vol. 2, pp. 105–253 (1976); and Erickson et al., The Proteins, 3rd ed., vol. 2, pp. 257–527 (1976). Solid phase synthesis is the preferred technique of making individual peptides since it is the most cost-effective method of making small peptides.

The peptides may also be made in transformed host cells using recombinant DNA techniques. To do so, a recombinant DNA molecule coding for the peptide is prepared. Methods of preparing such DNA and/or RNA molecules are well known in the art. For instance, sequences coding for the peptides could be excised from DNA using suitable restriction enzymes. The relevant sequences can be created using the polymerase chain reaction (PCR) with the inclusion of useful restriction sites for subsequent cloning. Alternatively, the DNA/RNA molecule could be synthesized using chemical synthesis techniques, such as the phosphoramidite method. Also, a combination of these techniques could be used.

The invention also includes a vector encoding the peptides in an appropriate host. The vector comprises the DNA molecule that encodes the peptides operatively linked to appropriate expression control sequences. Methods of effecting this operative linking, either before or after the peptide-encoding DNA molecule is inserted into the vector, are well known. Expression control sequences include promoters, activators, enhancers, operators, ribosomal binding sites, start signals, stop signals, cap signals, polyadenylation signals, and other signals involved with the control of transcription or translation.

The resulting vector comprising the peptide-encoding DNA molecule is used to transform an appropriate host. This transformation may be performed using methods well known in the art.

Any of a large number of available and well-known host cells may be used in the practice of this invention. The selection of a particular host is dependent upon a number of factors recognized by the art. These factors include, for example, compatibility with the chosen expression vector, toxicity to the host cell of the peptides encoded by the DNA molecule, rate of transformation, ease of recovery of the peptides, expression characteristics, bio-safety and costs. A balance of these factors must be struck with the understanding that not all hosts may be equally effective for the expression of a particular DNA sequence.

Within these general guidelines, useful microbial hosts include bacteria (such as *E. coli*), yeast (such as *Saccharomyces* sp. and *Pichia pastoris*) and other fungi, insects, plants, mammalian (including human) cells in culture, or other hosts known in the art.

Next, the transformed host is cultured under conventional fermentation conditions so that the desired peptides are expressed. Such fermentation conditions are well known in the art.

Finally, the peptides are purified from the fermentation culture or from the host cells in which they are expressed. These purification methods are also well known in the art.

Compounds that contain derivatized peptides or which contain non-peptide groups may be synthesized by well-known organic chemistry techniques.

Uses of the Compounds

The compounds of this invention have the ability to bind to and activate the c-MP1 receptor, and/or have the ability to stimulate the production (both in vivo and in vitro) of platelets ("thrombopoietic activity") and platelet precursors ("megakaryocytopoietic activity"). To measure the activity (-ies) of these compounds, one can utilize standard assays, such as those described in WO95/26746 entitled "Compositions and Methods for Stimulating Megakaryocyte Growth and Differentiation". In vivo assays are further described in the Examples section herein.

The conditions to be treated by the methods and compositions of the present invention are generally those which involve an existing megakaryocyte/platelet deficiency or an expected or anticipated megakaryocyte/platelet deficiency in the fuxture (e.g., because of planned surgery or platelet donation). Such conditions may be the result of a deficiency (temporary or permanent) of active Mp1 ligand in vivo. The generic term for platelet deficiency is thrombocytopenia, and hence the methods and compositions of the present invention are generally available for prophylactically or therapeutically treating thrombocytopenia in patients in need thereof.

The World Health Organization has classified the degree of thrombocytopenia on the number of circulating platelets in the individual (Miller, et al., Cancer 47:210–211 (1981)). For example, an individual showing no signs of thrombocytopenia (Grade 0) will generally have at least 100,000 platelets/mm^3. Mild thrombocytopenia (Grade 1) indicates a circulating level of platelets between 79,000 and 99,000/mm^3. Moderate thrombocytopenia (Grade 2) shows between 50,000 and 74,000 platelets/mm^3 and severe thrombocytopenia is characterized by between 25,000 and 49,000 platelets/mm^3. Life-threatening or debilitating thrombocytopenia is characterized by a circulating concentration of platelets of less than 25,000/mm^3.

Thrombocytopenia (platelet deficiencies) may be present for various reasons, including chemotherapy and other therapy with a variety of drugs, radiation therapy, surgery, accidental blood loss, and other specific disease conditions. Exemplary specific disease conditions that involve thrombocytopenia and may be treated in accordance with this invention are: aplastic anemia; idiopathic or immune thrombocytopenia (ITP), including idiopathic thrombocytopenic purpura associated with breast cancer; HIV associated ITP and HIV-related thrombotic thrombocytopenic purpura; metastatic tumors which result in thrombocytopenia; systemic lupus erythematosus; including neonatal lupus syndrome splenomegaly; Fanconi's syndrome; vitamin B12 deficiency; folic acid deficiency; May-Hegglin anomaly; Wiskott-Aldrich syndrome; chronic liver disease; myelodysplastic syndrome associated with thrombocytopenia; paroxysmal nocturnal hemoglobinuria; acute profound thrombocytopenia following C7E3 Fab (Abciximab) therapy; alloimmune thrombocytopenia, including maternal alloimmune thrombocytopenia; thrombocytopenia associated with antiphospholipid antibodies and thrombosis; autoimmune thrombocytopenia; drug-induced immune thrombocytopenia, including carboplatin-induced thrombocytopenia, heparin-induced thrombocytopenia; fetal thrombocytopenia; gestational thrombocytopenia; Hughes' syndrome; lupoid thrombocytopenia; accidental and/or massive blood loss; myeloproliferative disorders; thrombocytopenia in patients with malignancies; thrombotic thrombocytopenia purpura, including thrombotic microangiopathy manifesting as thrombotic thrombocytopenic purpura/hemolytic uremic syndrome in cancer patients; autoimmune hemolytic anemia; occult jejunal diverticulum perforation; pure red cell aplasia; autoimmune thrombocytopenia; nephropathia epidemica; rifampicin-associated acute renal failure; Paris-Trousseau thrombocytopenia; neonatal alloimmune thrombocytopenia; paroxysmal nocturnal hemoglobinuria; hematologic changes in stomach cancer; hemolytic uremic syndromes in childhood; hematologic manifestations related to viral infection including hepatitis A virus and CMV-associated thrombocytopenia. Also, certain treatments for AIDS result in thrombocytopenia (e.g., AZT). Certain wound healing disorders might also benefit from an increase in platelet numbers.

With regard to anticipated platelet deficiencies, e.g., due to future surgery, a compound of the present invention could be administered several days to several hours prior to the need for platelets. With regard to acute situations, e.g., accidental and massive blood loss, a compound of this invention could be administered along with blood or purified platelets.

The compounds of this invention may also be useful in stimulating certain cell types other than megakaryocytes if such cells are found to express Mpl receptor. Conditions associated with such cells that express the Mpl receptor, which are responsive to stimulation by the Mpl ligand, are also within the scope of this invention.

The compounds of this invention may be used in any situation in which production of platelets or platelet precursor cells is desired, or in which stimulation of the c-MP1 receptor is desired. Thus, for example, the compounds of this invention may be used to treat any condition in a manmal wherein there is a need of platelets, megakaryocytes, and the like. Such conditions are described in detail in the following exemplary sources: WO95/26746; WO95/21919; WO95/18858; WO95/21920 and are incorporated herein.

The compounds of this invention may also be useful in maintaining the viability or storage life of platelets and/or megakaryocytes and related cells. Accordingly, it could be useful to include an effective amount of one or more such compounds in a composition containing such cells.

By "mammal" is meant any mammal, including humans, domestic animals including dogs and cats; exotic and/or zoo animals including monkeys; laboratory animals including mice, rats, and guinea pigs; farm animals including horses, cattle, sheep, goats, and pigs; and the like. The preferred mammal is human.

Pharmaceutical Compositions

The present invention also provides methods of using pharmaceutical compositions of the inventive compounds. Such pharmaceutical compositions may be for administration for injection, or for oral, nasal, transdermal or other forms of administration, including, e.g., by intravenous, intradermal, intramuscular, intramammary, intraperitoneal, intrathecal, intraocular, retrobulbar, intrapulmonary (e.g., aerosolized drugs) or subcutaneous injection (including depot administration for long term release); by sublingual, anal, vaginal, or by surgical implantation, e.g., embedded

under the splenic capsule, brain, or in the cornea. The treatment may consist of a single dose or a plurality of doses over a period of time. In general, comprehended by the invention are pharmaceutical compositions comprising effective amounts of a compound of the invention together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer content (e.g., Tris-HCl, acetate, phosphate), pH and ionic strength; additives such as detergents and solubilizing agents (e.g., Tween 80, Polysorbate 90), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., Thimersol, benzyl alcohol) and bulking substances (e.g., lactose, mannitol); incorporation of the material into particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc. or into liposomes. Hyaluronic acid may also be used, and this may have the effect of promoting sustained duration in the circulation. The pharmaceutical compositions optionally may include still other pharmaceutically acceptable liquid, semisolid, or solid diluents that serve as pharmaceutical vehicles, excipients, or media, including but are not limited to, polyoxyethylene sorbitan monolaurate, magnesium stearate, methyl- and propylhydroxybenzoate, starches, sucrose, dextrose, gum acacia, calcium phosphate, mineral oil, cocoa butter, and oil of theobroma. Such compositions may influence the physical state, stability, rate of in vivo release, and rate of in vivo clearance of the present proteins and derivatives. See, e.g., Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, Pa. 18042) pages 1435–1712 which are herein incorporated by reference. The compositions may be prepared in liquid form, or may be in dried powder, such as lyophilized form. Implantable sustained release formulations are also contemplated, as are transdermal formulations.

Contemplated for use herein are oral solid dosage forms, which are described generally in Remington's Pharmaceutical Sciences, 18th Ed. 1990 (Mack Publishing Co. Easton Pa. 18042) at Chapter 89, which is herein incorporated by reference. Solid dosage forms include tablets, capsules, pills, troches or lozenges, cachets or pellets. Also, liposomal or proteinoid encapsulation may be used to formulate the present compositions (as, for example, proteinoid microspheres reported in U.S. Pat. No. 4,925,673). Liposomal encapsulation may be used and the liposomes may be derivatized with various polymers (e.g., U.S. Pat. No. 5,013, 556). A description of possible solid dosage forms for the therapeutic is given by Marshall, K., Modern Pharmaceutics, Edited by G. S. Banker and C. T. Rhodes Chapter 10, 1979, herein incorporated by reference. In general, the formulation will include the inventive compound, and inert ingredients which allow for protection against the stomach environment, and release of the biologically active material in the intestine.

Also specifically contemplated are oral dosage forms of the above inventive compounds. If necessary, the compounds may be chemically modified so that oral delivery is efficacious. Generally, the chemical modification contemplated is the attachment of at least one moiety to the compound molecule itself, where said moiety permits (a) inhibition of proteolysis; and (b) uptake into the blood stream from the stomach or intestine. Also desired is the increase in overall stability of the compound and increase in circulation time in the body. Examples of such moieties include: Polyethylene glycol, copolymers of ethylene glycol and propylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone and polyproline (Abuchowski and Davis, Soluble Polymer-Enzyme Adducts, Enzymes as Drugs, Hocenberg and Roberts, eds., Wiley-Interscience, New York, N.Y., (1981), pp 367–383; Newmark, et al., J. Appl. Biochem. 4:185–189 (1982)). Other polymers that could be used are poly-1,3-dioxolane and poly-1,3,6-tioxocane. Preferred for pharmaceutical usage, as indicated above, are polyethylene glycol moieties.

For the oral delivery dosage forms, it is also possible to use a salt of a modified aliphatic amino acid, such as sodium N-(8-[2-hydroxybenzoyl] amino) caprylate (SNAC), as a carrier to enhance absorption of the therapeutic compounds of this invention. The clinical efficacy of a heparin formulation using SNAC has been demonstrated in a Phase II trial conducted by Emisphere Technologies. See U.S. Pat. No. 5,792,451, "Oral drug delivery composition and methods".

The therapeutic can be included in the formulation as fine multiparticulates in the form of granules or pellets of particle size about 1 mm. The formulation of the material for capsule administration could also be as a powder, lightly compressed plugs or even as tablets. The therapeutic could be prepared by compression.

Colorants and flavoring agents may all be included. For example, the protein (or derivative) may be formulated (such as by liposome or microsphere encapsulation) and then further contained within an edible product, such as a refrigerated beverage containing colorants and flavoring agents.

One may dilute or increase the volume of the therapeutic with an inert material. These diluents could include carbohydrates, especially mannitol, α-lactose, anhydrous lactose, cellulose, sucrose, modified dextrans and starch. Certain inorganic salts may also be used as fillers including calcium triphosphate, magnesium carbonate and sodium chloride. Some commercially available diluents are Fast-Flo, Emdex, STA-Rx 1500, Emcompress and Avicell.

Disintegrants may be included in the formulation of the therapeutic into a solid dosage form. Materials used as disintegrants include but are not limited to starch including the commercial disintegrant based on starch, Explotab. Sodium starch glycolate, Amberlite, sodium carboxymethylcellulose, ultramylopectin, sodium alginate, gelatin, orange peel, acid carboxymethyl cellulose, natural sponge and bentonite may all be used. Another form of the disintegrants are the insoluble cationic exchange resins. Powdered gums may be used as disintegrants and as binders and these can include powdered gums such as agar, Karaya or tragacanth. Alginic acid and its sodium salt are also useful as disintegrants.

Binders may be used to hold the therapeutic agent together to form a hard tablet and include materials from natural products such as acacia, tragacanth, starch and gelatin. Others include methyl cellulose (MC), ethyl cellulose (EC) and carboxymethyl cellulose (CMC). Polyvinyl pyrrolidone (PVP) and hydroxypropylmethyl cellulose (HPMC) could both be used in alcoholic solutions to granulate the therapeutic.

An antifrictional agent may be included in the formulation of the therapeutic to prevent sticking during the formulation process. Lubricants may be used as a layer between the therapeutic and the die wall, and these can include but are not limited to; stearic acid including its magnesium and calcium salts, polytetrafluoroethylene (PTFE), liquid paraffin, vegetable oils and waxes. Soluble lubricants may also be used such as sodium lauryl sulfate, magnesium lauryl sulfate, polyethylene glycol of various molecular weights, Carbowax 4000 and 6000.

Glidants that might improve the flow properties of the drug during formulation and to aid rearrangement during compression might be added. The glidants may include starch, talc, pyrogenic silica and hydrated silicoaluminate.

To aid dissolution of the therapeutic into the aqueous environment, a surfactant might be added as a wetting agent. Surfactants may include anionic detergents such as sodium lauryl sulfate, dioctyl sodium sulfosuccinate and dioctyl sodium sulfonate. Cationic detergents might be used and could include benzalkonium chloride or benzethonium chloride. The list of potential nonionic detergents that could be included in the formulation as surfactants are lauromacrogol 400, polyoxyl 40 stearate, polyoxyethylene hydrogenated castor oil 10, 50 and 60, glycerol monostearate, polysorbate 40, 60, 65 and 80, sucrose fatty acid ester, methyl cellulose and carboxymethyl cellulose. These surfactants could be present in the formulation of the protein or derivative either alone or as a mixture in different ratios.

Additives which potentially enhance uptake of the compound are for instance the fatty acids oleic acid, linoleic acid and linolenic acid.

Controlled release fomnulation may be desirable. The drug could be incorporated into an inert matrix which permits release by either diffusion or leaching mechanisms e.g., gums. Slowly degenerating matrices may also be incorporated into the formulation, e.g., alginates, polysaccharides. Another form of a controlled release of this therapeutic is by a method based on the Oros therapeutic system (Alza Corp.), i.e., the drug is enclosed in a semipermeable membrane which allows water to enter and push drug out through a single small opening due to osmotic effects. Some enteric coatings also have a delayed release effect.

Other coatings may be used for the formulation. These include a variety of sugars which could be applied in a coating pan. The therapeutic agent could also be given in a film coated tablet and the materials used in this instance are divided into 2 groups. The first are the nonenteric materials and include methyl cellulose, ethyl cellulose, hydroxyethyl cellulose, methylhydroxy-ethyl cellulose, hydroxypropyl cellulose, hydroxypropyl-methyl cellulose, sodium carboxymethyl cellulose, providone and the polyethylene glycols. The second group consists of the enteric materials that are commonly esters of phthalic acid.

A mix of materials might be used to provide the optimum film coating. Film coating may be carried out in a pan coater or in a fluidized bed or by compression coating.

Also contemplated herein is pulmonary delivery of the present protein (or derivatives thereof). The protein (or derivative) is delivered to the lungs of a mammal while inhaling and traverses across the lung epithelial lining to the blood stream. (Other reports of this include Adjei et al., Pharmaceutical Research 7:565–569 (1990); Adjei et al., International Journal of Pharmaceutics 63:135–144 (1990) (leuprolide acetate); Braquet et al., Journal of Cardiovascular Pharmacology 13 (suppl. 5): s. 143–146 (1989) (endothelin-1); Hubbard et al., Annals of Internal Medicine 3:206–212 (1989)(α1-antitrypsin), Smith et al., J. Clin. Invest. 84:1145–1146 (1989)(α1-proteinase); Oswein et al., "Aerosolization of Proteins", Proceedings of Symposium on Respiratory Drug Delivery II, Keystone, Colo., March, 1990 (recombinant human growth hormone); Debs et al., The Journal of Immunology 140:3482–3488 (1988)(interferon-γ and tumor necrosis factor α) and Platz et al., U.S. Pat. No. 5,284,656 (granulocyte colony stimulating factor).

Contemplated for use in the practice of this invention are a wide range of mechanical devices designed for pulmonary delivery of therapeutic products, including but not limited to nebulizers, metered dose inhalers, and powder inhalers, all of which are familiar to those skilled in the art.

Some specific examples of commercially available devices suitable for the practice of this invention are the Ultravent nebulizer, manufactured by Mallinckrodt, Inc., St. Louis, Mo.; the Acorn II nebulizer, manufactured by Marquest Medical Products, Englewood, Colo.; the Ventolin metered dose inhaler, manufactured by Glaxo Inc., Research Triangle Park, N.C.; and the Spinhaler powder inhaler, manufactured by Fisons Corp., Bedford, Mass.

All such devices require the use of formulations suitable for the dispensing of the inventive compound. Typically, each formulation is specific to the type of device employed and may involve the use of an appropriate propellant material, in addition to diluents, adjuvants and/or carriers useful in therapy.

The inventive compound should most advantageously be prepared in particulate form with an average particle size of less than 10 μm (or microns), most preferably 0.5 to 5 μm, for most effective delivery to the distal lung.

Carriers include carbohydrates such as trehalose, mannitol, xylitol, sucrose, lactose, and sorbitol. Other ingredients for use in formulations may include DPPC, DOPE, DSPC and DOPC. Natural or synthetic surfactants may be used. Polyethylene glycol may be used (even apart from its use in derivatizing the protein or analog). Dextrans, such as cyclodextran, may be used. Bile salts and other related enhancers may be used. Cellulose and cellulose derivatives may be used. Amino acids may be used, such as use in a buffer formulation.

Also, the use of liposomes, microcapsules or microspheres, inclusion complexes, or other types of carriers is contemplated.

Formulations suitable for use with a nebulizer, either jet or ultrasonic, will typically comprise the inventive compound dissolved in water at a concentration of about 0.1 to 25 mg of biologically active protein per mL of solution. The formulation may also include a buffer and a simple sugar (e.g., for protein stabilization and regulation of osmotic pressure). The nebulizer formulation may also contain a surfactant, to reduce or prevent surface induced aggregation of the protein caused by atomization of the solution in forming the aerosol.

Formulations for use with a metered-dose inhaler device will generally comprise a finely divided powder containing the inventive compound suspended in a propellant with the aid of a surfactant. The propellant may be any conventional material employed for this purpose, such as a chlorofluorocarbon, a hydrochlorofluorocarbon, a hydrofluorocarbon, or a hydrocarbon, including trichlorofluoromethane, dichlorodifluoromethane, dichlorotetrafluoroethanol, and 1,1,1,2-tetrafluoroethane, or combinations thereof. Suitable surfactants include sorbitan trioleate and soya lecithin. Oleic acid may also be useful as a surfactant.

Formulations for dispensing from a powder inhaler device will comprise a finely divided dry powder containing the inventive compound and may also include a bulking agent, such as lactose, sorbitol, sucrose, mannitol, trehalose, or xylitol in amounts which facilitate dispersal of the powder from the device, e.g., 50 to 90% by weight of the formulation.

Nasal delivery of the inventive compound is also contemplated. Nasal delivery allows the passage of the protein to the blood stream directly after administering the therapeutic product to the nose, without the necessity for deposition of the product in the lung. Formulations for nasal delivery include those with dextran or cyclodextran. Delivery via transport across other mucous membranes is also contemplated.

Dosages

The dosage regimen involved in a method for treating the above-described conditions will be determined by the attending physician, considering various factors which modify the action of drugs, e.g. the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. Generally, the dose should be in the range of 0.1 μg to 100 mg of the inventive compound per kilogram of body weight per day, preferably 0.1 to 1000 μg/kg; and more preferably 0.1 to 150 μg/kg, given in daily doses or in equivalent doses at longer or shorter intervals, e.g., every other day, twice weekly, weekly, or twice or three times daily.

The inventive compound may be administered by an initial bolus followed by a continuous infusion to maintain therapeutic circulating levels of drug product. As another example, the inventive compound may be administered as a one-time dose. Those of ordinary skill in the art will readily optimize effective dosages and administration regimens as determined by good medical practice and the clinical condition of the individual patient. The frequency of dosing will depend on the pharmacokinetic parameters of the agents and the route of administration. The optimal pharmaceutical formulation will be determined by one skilled in the, art depending upon the route of administration and desired dosage. See for example, Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, Pa. 18042) pages 1435–1712, the disclosure of which is hereby incorporated by reference. Such formulations may influence the physical state, stability, rate of in vivo release, and rate of in vivo clearance of the administered agents. Depending on the route of administration, a suitable dose may be calculated according to body weight, body surface area or organ size. Further refinement of the calculations necessary to determine the appropriate dosage for treatment involving each of the above mentioned formulations is routinely made by those of ordinary skill in the art without undue experimentation, especially in light of the dosage information and assays disclosed herein, as well as the pharmacokinetic data observed in the human clinical trials discussed above. Appropriate dosages may be ascertained through use of established assays for determining blood levels dosages in conjunction with appropriate dose-response data. The final dosage regimen will be determined by the attending physician, considering various factors which modify the action of drugs, e.g. the drug's specific activity, the severity of the damage and the responsiveness of the patient, the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. As studies are conducted, further information will emerge regarding the appropriate dosage levels and duration of treatment for various diseases and conditions.

The therapeutic methods, compositions and compounds of the present invention may also be employed, alone or in combination with other cytokines, soluble Mpl receptor, hematopoietic factors, interleukins, growth factors or antibodies in the treatment of disease states characterized by other symptoms as well as platelet deficiencies. It is anticipated that the inventive compound will prove useful in treating some forms of thrombocytopenia in combination with general stimulators of hematopoiesis, such as IL-3 or GM-CSF. Other megakaryocytic stimulatory factors, i.e., meg-CSF, stem cell factor (SCF), leukemia inhibitory factor (LIF), oncostatin M (OSM), or other molecules with megakaryocyte stimulating activity may also be employed with Mpl ligand. Additional exemplary cytokines or hematopoietic factors for such co-administration include IL-1 alpha, IL-1 beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-11, colony stimulating factor-1 (CSF-1), M-CSF, SCF, GM-CSF, granulocyte colony stimulating factor (G-CSF), EPO, interferon-alpha (IFN-alpha), consensus interferon, IFN-beta, IFN-gamrna, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, thrombopoietin (TPO), angiopoietins, for example Ang-1, Ang-2, Ang4, Ang-Y, the human angiopoietin-like polypeptide, vascular endothelial growth factor (VEGF), angiogenin, bone morphogenic protein-1, bone morphogenic protein-2, bone morphogenic protein-3, bone morphogenic protein4, bone morphogenic protein-5, bone morphogenic protein-6, bone morphogenic protein-7, bone morphogenic protein-8, bone morphogenic protein-9, bone morphogenic protein-10, bone morphogenic protein-11, bone morphogenic protein-12, bone morphogenic protein-13, bone morphogenic protein-14, bone morphogenic protein-15, bone morphogenic protein receptor IA, bone morphogenic protein receptor IB, brain derived neurotrophic factor, ciliary neutrophic factor, ciliary neutrophic factor receptor a, cytokine-induced neutrophil chemotactic factor 1, cytokine-induced neutrophil, chemotactic factor 2 α, cytokine-induced neutrophil chemotactic factor 2 β, β endothelial cell growth factor, endothelin 1, epidermal growth factor, epithelial-derived neutrophil attractant, fibroblast growth factor 4, fibroblast growth factor 5, fibroblast growth factor 6, fibroblast growth factor 7, fibroblast growth factor 8, fibroblast growth factor 8b, fibroblast growth factor 8c, fibroblast growth factor 9, fibroblast growth factor 10, fibroblast growth factor acidic, fibroblast growth factor basic, glial cell line-derived neutrophic factor receptor α 1, glial cell line-derived neutrophic factor receptor α 2, growth related protein, growth related protein α, growth related protein β, growth related protein γ, heparin binding epidermal growth factor, hepatocyte growth factor, hepatocyte growth factor receptor, insulin-like growth factor I, insulin-like growth factor receptor, insulin-like growth factor II, insulin-like growth factor binding protein, keratinocyte growth factor, leukemia inhibitory factor, leukemia inhibitory factor receptor α, nerve growth factor nerve growth factor receptor, neurotrophin-3, neurotrophin-4, placenta growth factor, placenta growth factor 2, platelet-derived endothelial cell growth factor, platelet derived growth factor, platelet derived growth factor A chain, platelet derived growth factor AA, platelet derived growth factor AB, platelet derived growth factor B chain, platelet derived growth factor BB, platelet derived growth factor receptor α, platelet derived growth factor receptor β, pre-B cell growth stimulating factor, stem cell factor receptor, TNF, including TNF0, TNF 1, TNF2, transforming growth factor α, transforring growth factor β, transforming growth factor β1, transforming growth factor β 1.2, transforming growth factor β2, transforming growth factor β3, transforming growth factor β5, latent transforming growth factor β1, transforming growth factor β binding protein I, transforming growth factor β binding protein II, transforming growth factor β, binding protein III, tumor necrosis factor receptor type I, tumor necrosis factor receptor type II, urokinase-type plasminogen activator receptor, vascular endothelial growth factor, and chimeric proteins and biologically or immunologically active fragments thereof. It may further be useful to administer, either simultaneously or sequentially, an effective amount of a soluble mammalian Mpl receptor, which appears to have an effect of causing megakaryocytes to fragment into platelets once the megakaryocytes have reached mature form. Thus, administration of an inventive compound (to enhance the number of mature megakaryocytes) followed by administration of the soluble

Mpl receptor (to inactivate the ligand and allow the mature megakaryocytes to produce platelets) is expected to be a particularly effective means of stimulating platelet production. The dosage recited above would be adjusted to compensate for such additional components in the therapeutic composition. Progress of the treated patient can be monitored by conventional methods.

In cases where the inventive compounds are added to compositions of platelets and/or megakaryocytes and related cells, the amount to be included will generally be ascertained experimentally by techniques and assays known in the art. An exemplary range of amounts is 0.1 μg −1 mg inventive compound per 10^6 cells.

It is understood that the application of the teachings of the present invention to a specific problem or situation will be within the capabilities of one having ordinary skill in the art in light of the teachings contained herein. Examples of the products of the present invention and representative processes for their isolation, use, and manufacture appear below.

EXAMPLES

I. The following sets forth exemplary methods for making some of the compounds of the first group disclosed herein.

A. Materials and Methods

All amino acid derivatives (all of L-configurations) and resins used in peptide synthesis were purchased from Novabiochem. Peptide synthesis reagents (DCC, HOBt, etc.) were purchased in the solution forms from Applied Biosystems, Inc. The two PEG derivatives were from Shearwater Polymers, Inc. All solvents (dichloromethane, N-methylpyrrolidinone, methanol, acetonitrile) were from EM Sciences. Analytical HPLC was run on a Beckman system with a Vydac column (0.46 cm×25 cm, C18 reversed phase, 5 mm), at a flow rate of 1 ml/min and with dual UV detection at 220 and 280 nm. Linear gradients were used for all HPLC operations with two mobile phases: Buffer A—H_2O (0.1% TFA) and Buffer B-acetonitrile (0.1% TFA). The numbered peptides referred to herein, e.g., 17b, 18, 19, and 20, are numbered in reference to Table 1, and some of them are further illustrated in FIGS. 2 and 3.

Peptide synthesis. All peptides were prepared by the well established stepwise solid phase synthesis method. Solid-phase synthesis with Fmoc chemistry was carried out using an ABI Peptide Synthesizer. Typically, peptide synthesis began with a preloaded Wang resin on a 0.1 mmol scale. Fmoc deprotection was carried out with the standard piperidine protocol. The coupling was effected using DCC/HOBt. Side-chain protecting groups were: Glu(O-t-Bu), Thr(t-Bu), Arg(Pbf), Gln(Trt), Trp(t-Boc) and Cys(Trt). For the first peptide precursor for pegylation, Dde was used for side chain protection of the Lys on the linker and Boc-Ile-OH was used for the last coupling. Dde was removed by using anhydrous hydrazine (2% in NMP, 3×2 min), followed by coupling with bromoacetic anhydride preformed by the action of DCC. For peptide 18, the cysteine side chain in the linker was protected by a trityl group. The final deprotection and cleavage of all peptidyl-resins was effected at RT for 4 hr, using trifluoroacetic acid (TFA) containing 2.5% H_2O, 5% phenol, 2.5% triisopropylsilane and 2.5% thioanisole. After removal of TFA, the cleaved peptide was precipitated with cold anhydrous ether. Disulfide formation of the cyclic peptide was performed directly on the crude material by using 15% DMSO in H_2O (pH 7.5). All crude peptides were purified by preparative reverse phase HPLC and the structures were confirmed by ESI-MS and amino acid analysis.

Alternatively, all peptides described above could also be prepared by using the t-Boc chemistry. In this case, the starting resins would be the classic Merrifield or Pam resin, and side chain protecting groups would be: Glu(OBzl), Thr(Bzl), Arg(Tos), Trp(CHO), Cys(p-MeBzl). Hydrogen fluoride (HF) would be used for the final cleavage of the peptidyl resins.

All the tandem dimeric peptides described in this study that have linkers composed of natural amino acids can also be prepared by recombinant DNA technology.

PEGylation. A novel, convergent strategy for the pegylation of synthetic peptides was developed which consists of combining, through forming a conjugate linkage in solution, a peptide and a PEG moiety, each bearing a special functionality that is mutually reactive toward the other. The precursor peptides can be easily prepared with the conventional solid phase synthesis as described above. As described below, these peptides are "preactivated" with an appropriate functional group at a specific site. The precursors are purified and fully characterized prior to reacting with the PEG moiety. Ligation of the peptide with PEG usually takes place in aqueous phase and can be easily monitored by reverse phase analytical HPLC. The pegylated peptides can be easily purified by preparative HPLC and characterized by analytical HPLC, amino acid analysis and laser desorption mass spectrometry.

Preparation of peptide 19. Peptide 17b (12 mg) and MeO-PEG-SH 5000 (30 mg, 2 equiv.) were dissolved in 1 ml aqueous buffer (pH 8). The mixture was incubated at RT for about 30 min and the reaction was checked by analytical HPLC which showed a >80% completion of the reaction. The pegylated material was isolated by preparative HPLC.

Preparation of peptide 20. Peptide 18 (14 mg) and MeO-PEG-maleimide (25 mg) were dissolved in about 1.5 ml aqueous buffer (pH 8). The mixture was incubated at RT for about 30 min, at which time ~70% transformation was complete as monitored with analytical HPLC by applying an aliquot of sample to the HPLC column. The pegylated material was purified by preparative HPLC.

Bioactivity assay. The TPO in vitro bioassay is a mitogenic assay utilizing an IL-3 dependent clone of murine 32D cells that have been transfected with human Mpl receptor. This assay is described in greater detail in WO 95/26746. Cells are maintained in MEM medium containing 10% Fetal Clone II and 1 ng/ml mIL-3. Prior to sample addition, cells are prepared by rinsing twice with growth medium lacking mIL-3. An extended twelve point TPO standard curve is prepared, ranging from 3333 to 39 pg/ml. Four dilutions, estimated to fall within the linear portion of the standard curve, (1000 to 125 pg/ml), are prepared for each sample and run in triplicate. A volume of 100 μl of each dilution of sample or standard is added to appropriate wells of a 96 well microtiter plate containing 10,000 cells/well. After forty-four hours at 37° C. and 10% CO_2, MTS (a tetrazolium compound which is bioreduced by cells to a formazan) is added to each well. Approximately six hours later, the optical density is read on a plate reader at 490 nm. A dose response curve (log TPO concentration vs. O.D.-Background) is generated and linear regression analysis of points which fall in the linear portion of the standard curve is performed. Concentrations of unknown test samples are determined using the resulting linear equation and a correction for the dilution factor.

Abbreviations. HPLC: high performance liquid chromatography; ESI-MS: Electron spray ionization mass spectrometry; MALDI-MS: Matrix-assisted laser desorption ionization mass spectrometry; PEG: Poly(ethylene glycol). All amino acids are represented in the standard three-letter or single-letter codes, t-Boc: tert-Butoxycarbonyl; tBu: tert-

Butyl; Bzl: Benzyl; DCC: Dicylcohexylcarbodiimide; HOBt: I-Hydroxybenzotriazole; NMP: N-methyl-2-pyrrolidinone; Pbf: 2,2,4,6,7-pendamethyldihydrobenzofuran-5-sulfonyl; Trt: trityl; Dde: 1-(4,4-dimethyl-2,6-dioxo-cyclohexylidene)ethyl.

B. Results

TMP tandem dimers with polyglycine linkers. The design of sequentially linked TMP dimers was based on the assumption that a dimeric form of TMP was required for its effective interaction with c-MP1 (the TPO receptor) and that depending on how they were wound up against each other in the receptor context, the two TMP molecules could be tethered together in the C- to N-terminus configuration in a way that would not perturb the global dimeric conformation. Clearly, the activity of the tandem linked dimers may also depend on proper selection of the length and composition of the linker that joins the C- and -termini of the two sequentially aligned TMP monomers. Since no structural information of the TMP bound to c-MP1 was available, a series of dimeric peptides with linkers composed of 0 to 10 and 14 glycine residues (Table 1) were synthesized. Glycine was chosen because of its simplicity and flexibility. It was reasoned that a flexible polyglycine peptide chain might allow for the free folding of the two tethered TMP repeats into the required conformation, while more sterically hindered amino acid sequences may adopt undesired secondary structures whose rigidity might disrupt the correct packing of the dimeric peptide in the receptor context.

The resulting peptides are readily accessible by conventional solid phase peptide synthesis methods (Merrifiled, R. B., Journal of the American Chemical Society 85:2149 (1963)) with either Fmoc or t-Boc chemistry. Unlike the synthesis of the C-terminally linked parallel dimer (SEQ ID NO: 2) which required the use of an orthogonally protected lysine residue as the initial branch point to build the two peptide chains in a pseudosymmetrical way (Cwirla, S. E. et al., Science 276:1696–1699 (1997)), the synthesis of our tandem dimers was a straightforward, stepwise assembly of the continuous peptide chains from the C- to N-terminus. Since dimerization of TMP had a more dramatic effect on the proliferative activity than binding affinity as shown for the C-terminal dimer (Cwirla, S. E. et al., Science 276:1696–1699 (1997)), the synthetic peptides were tested directly for biological activity in a TPO-dependent cell-proliferation assay using an IL-3 dependent clone of murine 32D cells transfected with the full-length c-MP1 (Palacios, R. et al., Cell 41:727 (1985)). As the test results showed (see Table 1 below), all of the polyglycine linked tandem dimers demonstrated >1000 fold increases in potency as compared to the monomer, and were even more potent than the C-terminal dimer in this cell proliferation assay. The absolute activity of the C-terminal dimer in our assay was lower than that of the native TPO protein, which is different from the previously reported findings in which the C-terminal dimer was found to be as active as the natural ligand (Cwirla, S. E. et al., Science 276:1696–1699 (1997)). This might be due to differences in the conditions used in the two assays. Nevertheless, the difference in activity between tandem dimers © terminal of first monomer linked to N terminal of second monomer) and parallel dimers © terminal of first monomer linked to C terminal of second monomer) in the same assay clearly demonstrated the superiority of tandem dimerized product compared to parallel dimer products. It is interesting to note that a wide range of length is tolerated by the linker. The optimal linker with the selected TMP monomers (SEQ ID NO: 1) apparently is composed of 8 glycines.

Other tandem dimers. Subsequent to this first series of TMP tandem dimers, several other molecules were designed either with different linkers or containing modifications within the monomer itself. The first of these molecules, peptide 13, has a linker composed of GPNG, a sequence known to have a high propensity to form a β-turn-type secondary structure. Although still about 100-fold more potent than the monomer, this peptide was found to be >10-fold less active than the GGGG-linked analog. Thus, introduction of a relatively rigid β-turn at the linker region seemed to cause a slight distortion of the optimal agonist conformation in this short linker form.

The Trp9 in the TMP sequence is a highly conserved residue among the active peptides isolated from random peptide libraries. There is also a highly conserved Trp in the consensus sequences of EPO mimetic peptides and this Trp residue was found to be involved in the formation of a hydrophobic core between the two EPO Mimetic Peptides (EMPs) and contributed to hydrophobic interactions with the EPO receptor (Livnah, O. et al., Science 273:464–471 (1996)). By analogy, it was thought that the Trp9 residue in TMP might have a similar function in dimerization of the peptide ligand, and in an attempt to modulate and estimate the effects of noncovalent hydrophobic forces exerted by the two indole rings, several analogs were constructed resulting from mutations at the Trp. So in peptide 14, the Trp residue in each of the two TMP monomers was replaced with a Cys, and an intramolecular disulfide bond was formed between the two cysteines by oxidation which was envisioned to mimic the hydrophobic interactions between the two Trp residues in peptide dimerization. Peptide 15 is the reduced form of peptide 14. In peptide 16, the two Trp residues were replaced by Ala. As the assay data show, all three analogs were inactive. These data further demonstrated that Trp is important for the activity of the TPO mimetic peptide, not just for dimer formation.

The next two peptides (peptide 17a, and 18) each contain in their 8-amino acid linker a Lys or Cys residue. These two compounds are precursors to the two pegylated peptides (peptide 19 and 20) in which the side chain of the Lys or Cys is modified by a polyethylene glycol (PEG) moiety. It was decided to introduce a PEG moiety in the middle of a relatively long linker, so that the large PEG component (5 kDa) is far enough away from the important binding sites in the peptide molecule. PEG is a known biocompatible polymer which is increasingly used as a covalent modifier to improve the pharmacokinetic profiles of peptide- and protein-based therapeutics.

A modular, solution based method was devised for convenient pegylation of synthetic or recombinant peptides. The method is based on the now well established chemoselective ligation strategy which utilizes the specific reaction between a pair of mutually reactive functionalities. So, for pegylated peptide 19, the lysine side chain was preactivated with a bromoacetyl group to give peptide 17b to accommodate reaction with a thiol-derivatized PEG. To do that, an orthogonal protecting group, Dde, was employed for the protection of the lysine ε-amine. Once the whole peptide chain was assembled, the N-terminal amine was reprotected with t-Boc. Dde was then removed to allow for the bromoacetylation. This strategy gave a high quality crude peptide which was easily purified using conventional reverse phase HPLC. Ligation of the peptide with the thiol-modified PEG took place in aqueous buffer at pH 8 and the reaction completed within 30 min. MALDI-MS analysis of the purified, pegylated material revealed a characteristic, bell-shaped spectrum with an increment of 44 Da between the adjacent peaks. For PEG-peptide 20, a cysteine residue was placed in the linker region and its side chain thiol group

would serve as an attachment site for a maleimide-containing PEG. Similar conditions were used for the pegylation of this peptide. As the assay data revealed, these two pegylated peptides had even higher in vitro bioactivity as compared to their unpegylated counterparts.

Peptide 21 has in its 8-amino acid linker a potential glycosylation motif, NGS. Since the exemplary tandem dimers are made up of natural amino acids linked by peptide bonds, expression of such a molecule in an appropriate eukaryotic cell system should produce a glycopeptide with the carbohydrate moiety added on the side chain carboxyamide of Asn. Glycosylation is a common post-translational modification process which can have many positive impacts on the biological activity of a given protein by increasing its aqueous solubility and in vivo stability. As the assay data show, incorporation of this glycosylation motif into the linker maintained high bioactivity. The synthetic precursor of the potential glycopeptide had in effect an activity comparable to that of the -$(Gly)_8$- linked analog. Once glycosylated, this peptide is expected to have the same order of activity as the pegylated peptides, because of the similar chemophysical properties exhibited by a PEG and a carbohydrate moiety.

The last peptide is a dimer of a dimer. It was prepared by oxidizing peptide 18, which formed an intermolecular disulfide bond between the two cysteine residues located at the linker. This peptide was designed to address the possibility that TMP was active as a tetramer. The assay data showed that this peptide was not more active than an average tandem dimer on an adjusted molar basis, which indirectly supports the idea that the active form of TMP is indeed a dimer, otherwise dimerization of a tandem dimer would have a further impact on the bioactivity.

The following Table I summarizes relative activities of the above-described compounds in terms of the EC50 based on in vitro assays as described above.

TABLE I

	Compound	Relative Potency
	TPO	4.0
	TMP monomer (SEQ ID NO: 1)	1.0
	TMP C-C dimer (SEQ ID NO: 2)	3.5
	TMP-$(Gly)_n$-TMP:	
1	n = 0	4.5
2	n = 1	4.0
3	n = 2	4.0
4	n = 3	4.0
5	n = 4	4.0
6	n = 5	4.0
7	n = 6	4.0
8	n = 7	4.0
9	n = 8	4.5
10	n = 9	4.0
11	n = 10	4.0
12	n = 14	4.0
13	TMP-GPNG-TMP (SEQ ID NO. 10)	3.0
14	IEGPTLRQCLAARA-GGGGGGGG-IEGPTLRQCLAARA (SEQ ID NO. 11)	0.5
15	IEGPTLRQCLAARA-GGGGGGGG-IEGPTLRQCLAARA (SEQ ID NO. 12)	0.5
16	IEGPTLRQALAARA-GGGGGGGG-IEGPTLRQALAARA (SEQ ID NO. 13)	0.5
17a	TMP-GGGKGGGG-TMP (SEQ ID NO. 14)	4.0
17b	TMP-GGGK(BrAc)GGGG-TMP (SEQ ID NO. 15)	ND
18	TMP-GGGCGGGG-TMP (SEQ ID NO. 16)	4.0
19	TMP-GGGK(PEG)GGGG-TMP (SEQ ID NO. 17)	5.0
20	TMP-GGGC(PEG)GGGG-TMP (SEQ ID NO. 18)	5.0
21	TMP-GGGNGSGG-TMP (SEQ ID NO. 19)	4.0
22	TMP-GGGCGGGG-TMP TMP-GGGCGGGG-TMP (SEQ ID NO. 20)	4.0

NOTE:
In Table 1, numerals indicate approximately 1 log of activity, so that the difference in activity between "1" and "4" is approximately 1000-fold. An increment of 0.5 is an intermediate point, so that the difference in activity between "1" and "3.5" is approximately 500-fold. "ND" means not determined.

II. The following sets forth exemplary methods for making some of the compounds of the second group disclosed herein.

A. Preparation of an Fc fusion compound of the type shown in FIG. 6C.

A DNA sequence coding for the Fc region of human IgG1 fused in-frame to a dimer of the TPO-mimetic peptide (SEQ ID NO: 34) was placed under control of the luxPR promoter in the plasmid expression vector pAMG21 as follows.

The fusion gene was constructed using standard PCR technology. Templates for PCR reactions were the fusion vector containing the Fc sequence and a synthetic gene encoding the remainder of the compound of SEQ ID NO: 34.

The synthetic gene was constructed from the 4 overlapping oligonucleotides shown below:

1830-52 AAA GGT GGA GGT GGT GGT ATC GAA GGT CCG ACT CTG CGT CAG TGG CTG GCT GCT CGT GCT (SEQ ID NO: 35)

1830-53 ACC TCC ACC ACC AGC ACG AGC AGC CAG CCA CTG ACG CAG AGT CGG ACC (SEQ ID NO: 36)

1830-54 GGT GGT GGA GGT GGC GGC GGA GGT ATT GAG GGC CCA ACC CTT CGC CAA TGG CTT GCA GCA CGC GCA (SEQ ID NO: 37)

1830-55 AAA AAA AGG ATC CTC GAG ATT ATG CGC GTG CTG CAA GCC ATT GGC GAA GGG TTG GGC CCT CAA TAC CTC CGC CGC C (SEQ ID NO. 38)

The 4 oligonucleotides were annealed to form the duplex shown below:

```
AAAGGTGGAGGTGGTGGTATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCTGCTCGTGCT
1--------+---------+---------+---------+---------+---------+    60
                          CCAGGCTGAGACGCAGTCACCGACCGACGAGCACGA
 K  G  G  G  G  G  I  E  G  P  T  L  R  Q  W  L  A  A  R  A

GGTGGTGGAGGTGGCGGCGGAGGTATTGAGGGCCCAACCCTTCGCCAATGGCTTGCAGCA
---------+---------+---------+---------+---------+---------+   120
CCACCACCTCCACCGCCGCCTCCATAACTCCCGGGTTGGGAAGCGGTTACCGAACGTCGT
 G  G  G  G  G  G  G  G  I  E  G  P  T  L  R  Q  W  L  A  A

CGCGCA
------------------------------148
GCGCGTATTAGAGCTCCTAGGAAAAAAA
 R  A  *
```

SEQ ID NO: 39 [co-linear oligonucleotides 1830–52 and 1830–54]

SEQ ID NO: 40 [co-linear oligonucleotides 1830–53 and 1830–55]

and SEQ ID NO: 41 [the encoded amino acid sequence]

This duplex was amplified in a PCR reaction using 1830–52 and 1830–55 as the sense and antisense primers.

The Fc portion of the molecule was generated in a PCR reaction with Fc DNA using the primers

1216-52 AAC ATA AGT ACC TGT AGG ATC G (SEQ D NO: 42)

1830-51 TTCGATACCACCACCTCCACCTTTACCCGGAG-ACAGGGAGAGGCTCTTCTGC (SEQ ID NO: 43)

The oligonucleotides 1830–51 and 1830–52 contain an overlap of 24 nucleotides, allowing the two genes to be fused together in the correct reading frame by combining the above PCR products in a third reaction using the outside primers, 1216–52 and 1830–55.

The final PCR gene product (the full length fusion gene) was digested with restriction endonucleases XbaI and BamHI, and then ligated into the vector pAMG21 (see below), also digested with XbaI and BamHI. Ligated DNA was transformed into competent host cells of *E. coli* strain 2596 (GM221, described below). Clones were screened for the ability to produce the recombinant protein product and to possess the gene fusion having the correct nucleotide sequence. Protein expression levels were determined from 50 ml shaker flask studies. Whole cell lysates were analyzed for expression of the fusion via Coomassie stained PAGE gels.

The amino acid sequence of the fusion protein is shown below the corresponding nucleotide sequence:

```
XbaI
  |
  TCTAGATTTGTTTTAACTAATTAAAGGAGGAATAACATATGGACAAAACTCACACATGTC
1 ---------+---------+---------+---------+---------+---------+    60
  AGATCTAAACAAAATTGATTAATTTCCTCCTTATTGTATACCTGTTTTGAGTGTGTACAG
                                       M  D  K  T  H  T  C  P

  CACCTTGTCCAGCTCCGGAACTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAAC
61 ---------+---------+---------+---------+---------+---------+   120
  GTGGAACAGGTCGAGGCCTTGAGGACCCCCCTGGCAGTCAGAAGGAGAAGGGGGGTTTTG
    P  C  P  A  P  E  L  L  G  G  P  S  V  F  L  F  P  P  K  P

  CCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGA
121 ---------+---------+---------+---------+---------+---------+   180
  GGTTCCTGTGGGAGTACTAGAGGGCCTGGGGACTCCAGTGTACGCACCACCACCTGCACT
    K  D  T  L  M  I  S  R  T  P  E  V  T  C  V  V  V  D  V  S

  GCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATG
181 ---------+---------+---------+---------+---------+---------+   240
  CGGTGCTTCTGGGACTCCAGTTCAAGTTGACCATGCACCTGCCGCACCTCCACGTATTAC
    H  E  D  P  E  V  K  F  N  W  Y  V  D  G  V  E  V  H  N  A
```

-continued

```
    CCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCA
241 ---------+---------+---------+---------+---------+---------+  300
    GGTTCTGTTTCGGCGCCCTCCTCGTCATGTTGTCGTGCATGGCACACCAGTCGCAGGAGT
      K  T  K  P  R  E  E  Q  Y  N  S  T  Y  R  V  V  S  V  L  T

    CCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAG
301 ---------+---------+---------+---------+---------+---------+  360
    GGCAGGACGTGGTCCTGACCGACTTACCGTTCCTCATGTTCACGTTCCAGAGGTTGTTTC
      V  L  H  Q  D  W  L  N  G  K  E  Y  K  C  K  V  S  N  K  A

    CCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCAC
361 ---------+---------+---------+---------+---------+---------+  420
    GGGAGGGTCGGGGGTAGCTCTTTTGGTAGAGGTTTCGGTTTCCCGTCGGGGCTCTTGGTG
      L  P  A  P  I  E  K  T  I  S  K  A  K  G  Q  P  R  E  P  Q

    AGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCT
421 ---------+---------+---------+---------+---------+---------+  480
    TCCACATGTGGGACGGGGGTAGGGCCCTACTCGACTGGTTCTTGGTCCAGTCGGACTGGA
      V  Y  T  L  P  P  S  R  D  E  L  T  K  N  Q  V  S  L  T  C

    GCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGC
481 ---------+---------+---------+---------+---------+---------+  540
    CGGACCAGTTTCCGAAGATAGGGTCGCTGTAGCGGCACCTCACCCTCTCGTTACCCGTCG
      L  V  K  G  F  Y  P  S  D  I  A  V  E  W  E  S  N  G  Q  P

    CGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCT
541 ---------+---------+---------+---------+---------+---------+  600
    GCCTCTTGTTGATGTTCTGGTGCGGAGGGCACGACCTGAGGCTGCCGAGGAAGAAGGAGA
      E  N  N  Y  K  T  T  P  P  V  L  D  S  D  G  S  F  F  L  Y

    ACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCG
601 ---------+---------+---------+---------+---------+---------+  660
    TGTCGTTCGAGTGGCACCTGTTCTCGTCCACCGTCGTCCCCTTGCAGAAGAGTACGAGGC
      S  K  L  T  V  D  K  S  R  W  Q  Q  G  N  V  F  S  C  S  V

    TGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTA
661 ---------+---------+---------+---------+---------+---------+  720
    ACTACGTACTCCGAGACGTGTTGGTGATGTGCGTCTTCTCGGAGAGGGACAGAGGCCCAT
      M  H  E  A  L  H  N  H  Y  T  Q  K  S  L  S  L  S  P  G  K

    AAGGTGGAGGTGGTGGTATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCTGCTCGTGCTG
721 ---------+---------+---------+---------+---------+---------+  780
    TTCCACCTCCACCACCATAGCTTCCAGGCTGAGACGCAGTCACCGACCGACGAGCACGAC
      G  G  G  G  G  I  E  G  P  T  L  R  Q  W  L  A  A  R  A  G

    GTGGTGGAGGTGGCGGCGGAGGTATTGAGGGCCCAACCCTTCGCCAATGGCTTGCAGCAC
781 ---------+---------+---------+---------+---------+---------+  840
    CACCACCTCCACCGCCGCCTCCATAACTCCCGGGTTGGGAAGCGGTTACCGAACGTCGTG
      G  G  G  G  G  G  G  I  E  G  P  T  L  R  Q  W  L  A  A  R

              BamHI
                |
    GCGCATAATCTCGAGGATCCG
841 ---------+---------+-                                        861
    CGCGTATTAGAGCTCCTAGGC
      A
```

SEQ ID NO: 44 [single strand reading 5'-3' above],
SEQ ID NO: 45 [single strand reading 3'-5' above] and
SEQ ID NO: 46 [the encoded amino acid sequence]

pAMG21

The expression plasmid pAMG21 is available from the ATCC under accession number 98113, which was deposited on Jul. 24, 1996.

GM221 (Amgen Host Strain #2596)

The Amgen host strain #2596 is an *E. coli* K-12 strain that has been modified to contain both the temperature sensitive lambda repressor cI857s7 in the early ebg region and the $1acI^Q$ repressor in the late ebg region (68 minutes). The presence of these two repressor genes allows the use of this host with a variety of expression systems, however both of these repressors are irrelevant to the expression from $luxP_R$. The untransformed host has no antibiotic resistances.

The ribosome binding site of the cI857s7 gene has been modified to include an enhanced RBS. It has been inserted into the ebg operon between nucleotide position 1170 and 1411 as numbered in Genbank accession number M64441 $Gb_{13}Ba$ with deletion of the intervening ebg sequence.

The construct was delivered to the chromosome using a recombinant phage called MMebg-cI857s7 enhanced RBS #4 into F'tet/393. After recombination and resolution only the chromosomal insert described above remains in the cell. It was renamed F'tet/GM101.

F'tet/GM101 was then modified by the delivery of a $1acI^Q$ construct into the ebg operon between nucleotide position

2493 and 2937 as numbered in the Genbank accession number M64441Gb_Ba with the deletion of the intervening cbg sequence.

The construct was delivered to the chromosome using a recombinant phage called AGebg-LacIQ#5 into F'tet/GM101. After recombination and resolution only the chromosomal insert described above remains in the cell. It was renamed F'tet/GM221. The F'tet episome was cured from the strain using acridine orange at a concentration of 25 ug/ml in LB. The cured strain was identified as tetracyline sensitive and was stored as GM221.

The Fc fusion construct contained in plasmid pAMG21 (referred to herein as pAMG21-Fc-TMP-TMP), which in turn is contained in the host strain GM221 has been deposited at the ATCC under accession number 98957, with a deposit date of Oct. 22, 1998.

Expression. Cultures of pAMG21-Fc-TMP-TMP in *E. coli* GM221 in Luria Broth medium containing 50 μg/ml kanamycin were incubated at 37° C. prior to induction. Induction of Fc-TMP-TMP gene product expression from the luxPR promoter was achieved following the addition of the synthetic autoinducer N-(3-oxohexanoyl)-DL-homoserine lactone to the culture media to a final concentration of 20 ng/ml and cultures were incubated at 37° C. for a further 3 hours. After 3 hours, the bacterial cultures were examined by microscopy for the presence of inclusion bodies and were then collected by centrifugation. Refractile inclusion bodies were observed in induced cultures indicating that the Fc-TMP-TMP was most likely produced in the insoluble fraction in *E. coli*. Cell pellets were lysed directly by resuspension in Laemmili sample buffer containing 10% α-mercaptoethanol and were analyzed by SDS-PAGE. An intense Coomassie stained band of approximately 30 kDa was observed on an SDS-PAGE gel. The expected gene product would be 269 amino acids in length and have an expected molecular weight of about 29.5 kDa. Fermentation was also carried out under standard batch conditions at the 10 L scale, resulting in similar expression levels of the Fc-TMP-TMP to those obtained at bench scale.

Purification of Fc-TMP-TMP.

Cells were broken in water (1/10) by high pressure homogenization (2 passes at 14,000 PSI) and inclusion bodies were harvested by centrifugation (4200 RPM in J-6B for 1 hour). Inclusion bodies were solubilized in 6 M guanidine, 50 mM Tris, 8 mM DTT, pH 8.7 for 1 hour at a 1/10 ratio. The solubilized mixture was diluted 20 times into 2 M urea, 50 mM Tris, 160 mM arginine, 3 mM cysteine, pH 8.5. The mixture was stirred overnight in the cold. At this point in the procedure the Fc-TMP-TMP monomer subunits dimerize to form the disulfide-linked compound having the structure shown in FIG. 6C, and then concentrated about 10 fold by ultafiltration. It was then diluted 3 fold with 10 mM Tris, 1.5 M urea, pH 9. The pH of this mixture was then adjusted to pH 5 with acetic acid. The precipitate was removed by centrifugation and the supernatant was loaded onto a SP-Sepharose Fast Flow column equilibrated in 20 mM NaAc, 100 mM NaCl, pH 5(10 mg/ml protein load, room temperature). The protein was eluted off using a 20 column volume gradient in the same buffer ranging from 100 mM NaCl to 500 mM NaCl.

The pool from the column was diluted 3 fold and loaded onto a SP-Sepharose HP column in 20 mM NaAc, 150 mM NaCl, pH 5 (10 mg/ml protein load, room temperature). The protein was eluted off using a 20 column volume gradient in the same buffer ranging from 150 mM NaCl to 400 mM NaCl. The peak was pooled and filtered.

III. The following is a summary of in vivo data in mice with various compounds of this invention.

Mice. Normal female BDF1 approximately 10–12 weeks of age.

Bleed schedule. Ten mice per group treated on day 0, two groups started 4 days apart for a total of 20 mice per group. Five mice bled at each time point, mice were bled a minimum of three times a week. Mice were anesthetized with isoflurane and a total volume of 140–160 μl of blood was obtained by puncture of the orbital sinus. Blood was counted on a Technicon H1E blood analyzer running software for murine blood. Parameters measured were white blood cells, red blood cells, hematocrit, hemoglobin, platelets, neutrophils.

Treatments. Mice were either injected subcutaneously for a bolus treatment or implanted with 7 day micro-osmotic pumps for continuous delivery. Subcutaneous injections were delivered in a volume of 0.2 ml. Osmotic pumps were inserted into a subcutaneous incision made in the skin between the scapulae of anesthetized mice. Compounds were diluted in PBS with 0.1% BSA. All experiments included one control group, labeled "carrier" that were treated with this diluent only. The concentration of the test articles in the pumps was adjusted so that the calibrated flow rate from the pumps gave the treatment levels indicated in the graphs.

Compounds. A dose titration of the compound was delivered to mice in 7 day micro-osmotic pumps. Mice were treated with various compounds at a single dose of 100 ug/kg in 7 day osmotic pumps. Some of the same compounds were then given to mice as a single bolus injection.

Activity test results. The results of the activity experiments are shown in FIGS. 4 and 5. In dose response assays using 7-day micro-osmotic pumps (data not shown) the maximum effect was seen with the compound of SEQ ID NO: 18 was at 100 μg/kg/day; the 10 μg/kg/day dose was about 50% maximally active and 1 μg/kg/day was the lowest dose at which activity could be seen in this assay system. The compound at 10 μg/kg/day dose was about equally active as 100 μg/kg/day unpegylated rHu-MGDF in the same experiment.

IV. Discussion

It is well accepted that MGDF acts in a way similar to human growth hormone (hGH), i.e., one molecule of the protein ligand binds two molecules of the receptor for its activation (Wells, J. A. et al., Ann. Rev. Biochem. 65:609–634 (1996))). This interaction is mimicked by the action of the much smaller TMP peptide: However, the present studies suggest that this mimicry requires the concerted action of two TMP molecules, as covalent dimerization of TMP in either a C—C parallel or C—N sequential fashion increased the in vitro biological potency of the original monomer by a factor of greater than 10^3. The relatively low biopotency of the monomer is probably due to inefficient formation of the noncovalent dimer. A preformed covalent dimer has the ability to eliminate the entropy barrier for the formation of a noncovalent dimer which is exclusively driven by weak, noncovalent interactions between two molecules of the small, 14-residue peptide.

It is interesting to note that most of the tandem dimers are more potent than the C-terminal parallel dimers. Tandem dimenization seems to give the molecule a better fit conformation than does the C—C parallel dimerization. The seemingly unsymmetric feature of a tandem dimer might have brought it closer to the natural ligand which, as an unsymmetric molecule, uses two different sites to bind two identical receptor molecules.

Introduction of the PEG moiety was envisaged to enhance the in vivo activity of the modified peptide by providing it a protection against proteolytic degradation and by slowing down its clearance through renal filtration. It was unexpected that pegylation could further increase the in vitro bioactivity of a tandem dimerized TMP peptide in the cell-based proliferation assay.

V. The following is a summary of in vivo data in monkeys with various compounds of this invention.

In order to evaluate hematological parameters in female rhesus monkeys associated with administration of AMP2 via subcutaneous administration, the following protocol was designed and carried out. Five groups of three monkeys each were assembled. Group 1 served as control and received acetate buffer (20 mM sodium acetate, 0.25 M sodium chloride, pH 5) containing neither AMP2 nor pegylated, recombinant human MGDF (PEG-rHuMGDF). Group 2 received one or more dosage of AMP2 at intervals indicated below; Group 3 received 1000 μg/kg AMP2 at intervals indicated below; Group 4 received 5000 μg/kg AMP2 at intervals indicated below; and Group 5 received 100 μg/kg PEG-rHuMGDF at intervals indicated below.

The day on which the first single dose was administered was designated as Day 0 of Cycle 1. In Cycle 2, doses were administered on Days 21, 23, 25, 28, 30 and 32. During Cycle 3, a single dose was administered on Day 84, and in Cycle 4, a single dose was administration on Day 123. Animals were observed for clinical signs once daily during the acclimation period, three times daily (prior to dosing, immediately to 30 minutes following dosing, and 2 to 3 hours following dosing) on the dosing days, and once daily on the non-dosing days. Food consumption was calculated daily based on the number of food pieces given and the number left over for each animal from 7 days prior to the initiation of the dosing period to the end of the recovery period. Body weight for each animal was measured twice prior to the dosing regimen and twice during the dosing and recovery periods. Blood samples for hematology were prepared once prior to the initiation of dosing and once on Days 1, 3, 5, 7, 9, 11, 13, 15, 20, 22, 24, 26, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 55, 62, 69, 76, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 111, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 150. For pharmacokinetic analysis. 0.5 ml serum samples were collected once prior to dosing and once at 1, 4 and 24 hours after dosing. Samples were collected on Days 0, 21, 32, 84, and 123 and stored at approximately −70° C. until analysis. For antibody analysis, 2 ml blood samples were collected one week prior to the single dose and once on Days 0 (prior to dosing), 6, 13, 20, 27, 34, 41, 48, 55, 62, 69, 76, 83, 90, 97, 104, 111, 118, 129, 136, 143 and 150. Samples were stored at −70° C. until analysis.

Results indicated that platelet values increased in all treated groups with the largest increases seen in the PEG-rHuMGDF and high dose AMP2 groups.

In Cycle 1, peak platelet values increased approximately 3.3-fold and 3.1-fold in the PEG-rHuMGDF group (Day 9) and 5000 μg/kg AMP2 group (Day 9), respectively, compared to the mean platelet count in the control group. The low dose AMP2 platelet values increased approximately 1.5-fold higher than control on the same specified study days. Similar responses were noted in all other cycles.

However, in Cycle 4, the PEG-rHuMGDF group did not demonstrate as large of an increased platelet count as in the previous cycles. The PEG-rHuMGDF group has increased platelet counts of approximately 2-fold that in the control group 9 days after the dose of this cycle. For comparison, the mean platelet count in the highest dose AMP2 group in Cycle 4 was 3.3-fold higher than the control group. Additionally, PEG-rHuMGDF animals has a mean platelet count 53% lower than the control group mean platelet count at the start of Cycle 4 (per dose) and the mean platelet count for the group at the end of Cycle 4 *(27 days post dose) was 79% lower than that of the control group. For all AMP2 animals, the mean platelet counts at the start and end of Cycle 4 were ±15% of the platelet count in the control group.

In Cycle 1 and 2, a trend toward a decrease in red blood cell (RBC) counts was noted in all treated groups as compared to control. The decrease was most evident by Days 41 to 43 and the largest decrease in RBC was noted in the PEG-rHuMGDF group. The counts began returning to normal levels (as compared to control) as early as Day 47. The white blood cell (WBC) levels during Cycles 1 and 2 were dramatically increased (2.6-fold) as compared to control on Day 35. A slight increase was noted in the 5000 μg/kg AMP2 group on Day 33. Values headed toward normal (control) levels beginning on Day 37. A similar response was seen in Cycle 3 with no apparent change in WBC in Cycle 4 in any of the treated groups.

During Cycle 3, RBC counts were slightly decreased by Day 13 (following the single Cycle 3 dose) in all treated groups except for the 500 μg/kg AMP2 group. RBC values began returning to normal levels (as compared to control) by Day 17.

In Cycle 4, RBC counts decreased in all treated groups as compared to control except in the 500 μg/kg AMP2 group. Unlike the other cycles, there was more than one nadir present in this cycle. These decreases appeared from Day 1–9 post dose and began to recover as early as Day 11.

The results indicated that an increase in platelet counts, above that of control animals could be detected 7 to 9 days following dosing in all treated animals in all cycles tested. It appeared that the repeated dose phase caused a higher response in platelet production as compared to the single dose phases. By Cycle 4, the platelet response elicited by the PEG-rHuMGDF group was lower compared to the previous cycles and compared to that of the high dose AMP2 response. Decreases in RBC counts were noted in Cycles 1, 2, 3 and 4 in most treated groups at some point during each cycle of the study, however, all hematology parameters returned to normal levels (as compared to control) after dosing cessation.

Overall, these results indicated that treatment with AMP2 was well tolerated in the rhesus monkeys and that AMP2 resulted in increased platelet counts after various cycles of treatment. It did not appear, based on the platelet count results, that there was a biologically significant immune-mediated response to AMP2. In contrast, treatment in the various cycles with PEG-rHuMGDF did show an inhibition in platelet response by Cycle 4, suggesting that antibodies to PEG-rHuMGDF have been generated and these anti-MGDF antibodies may be crossreacting with endogenous rhesus TPO.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto, without departing from the spirit and scope of the invention as set forth herein.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 46

<210> SEQ ID NO 1
<211> LENGTH: 14
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: peptide

<400> SEQUENCE: 1

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala
1 5 10

<210> SEQ ID NO 2
<211> LENGTH: 14
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: peptide
<223> OTHER INFORMATION: Peptide is a subunit of a homodimer: Subunits in the dimer are covalently bonded at each carboxy terminus through peptide linkage with NH2-CH2-CH2-CH2-CH2-CH(CONH2)-NH-CO-CH2-CH2-NH2

<400> SEQUENCE: 2

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala
1 5 10

<210> SEQ ID NO 3
<211> LENGTH: 684
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: oligonucleotide

<400> SEQUENCE: 3

atggacaaaa ctcacacatg tccaccttgt ccagctccgg aactcctggg gggaccgtca 60

gtcttcctct tccccccaaa acccaaggac accctcatga tctcccggac ccctgaggtc 120

acatgcgtgg tggtggacgt gagccacgaa gaccctgagg tcaagttcaa ctggtacgtg 180

gacggcgtgg aggtgcataa tgccaagaca aagccgcggg aggagcagta caacagcacg 240

taccgtgtgg tcagcgtcct caccgtcctg caccaggact ggctgaatgg caaggagtac 300

aagtgcaagg tctccaacaa agccctccca gccccatcg agaaaaccat ctccaaagcc 360

aaagggcagc cccgagaacc acaggtgtac accctgcccc catcccggga tgagctgacc 420

aagaaccagg tcagcctgac ctgcctggtc aaaggcttct atcccagcga catcgccgtg 480

gagtgggaga gcaatgggca gccggagaac aactacaaga ccacgcctcc cgtgctggac 540

tccgacggct ccttcttcct ctacagcaag ctcaccgtgg acaagagcag gtggcagcag 600

gggaacgtct tctcatgctc cgtgatgcat gaggctctgc acaaccacta cacgcagaag 660

agcctctccc tgtctccggg taaa 684

<210> SEQ ID NO 4
<211> LENGTH: 684
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: oligonucleotide

<400> SEQUENCE: 4

-continued

tacctgtttt gagtgtgtac aggtggaaca ggtcgaggcc ttgaggaccc ccctggcagt 60

cagaaggaga agggggttt tgggttcctg tgggagtact agagggcctg gggactccag 120

tgtacgcacc accacctgca ctcggtgctt ctgggactcc agttcaagtt gaccatgcac 180

ctgccgcacc tccacgtatt acggttctgt ttcggcgccc tcctcgtcat gttgtcgtgc 240

atggcacacc agtcgcagga gtggcaggac gtggtcctga ccgacttacc gttcctcatg 300

ttcacgttcc agaggttgtt tcgggagggt cgggggtagc tcttttggta gaggtttcgg 360

tttcccgtcg gggctcttgg tgtccacatg tgggacgggg gtagggccct actcgactgg 420

ttcttggtcc agtcggactg gacggaccag tttccgaaga tagggtcgct gtagcggcac 480

ctcaccctct cgttacccgt cggcctcttg ttgatgttct ggtgcggagg gcacgacctg 540

aggctgccga ggaagaagga gatgtcgttc gagtggcacc tgttctcgtc caccgtcgtc 600

cccttgcaga agagtacgag gcactacgta ctccgagacg tgttggtgat gtgcgtcttc 660

tcggagaggg acagaggccc attt 684

<210> SEQ ID NO 5
<211> LENGTH: 228
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: peptide

<400> SEQUENCE: 5

Met Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
1 5 10 15

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
20 25 30

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
35 40 45

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
50 55 60

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr
65 70 75 80

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
85 90 95

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro
100 105 110

Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
115 120 125

Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val
130 135 140

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
145 150 155 160

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
165 170 175

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
180 185 190

Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
195 200 205

Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
210 215 220

Ser Pro Gly Lys
225

-continued

<210> SEQ ID NO 6
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: peptide

<400> SEQUENCE: 6

Gly Gly Gly Lys Gly Gly Gly Gly
1 5

<210> SEQ ID NO 7
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: peptide

<400> SEQUENCE: 7

Gly Gly Gly Asn Gly Ser Gly Gly
1 5

<210> SEQ ID NO 8
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: peptide

<400> SEQUENCE: 8

Gly Gly Gly Cys Gly Gly Gly Gly
1 5

<210> SEQ ID NO 9
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: peptide

<400> SEQUENCE: 9

Gly Pro Asn Gly
1

<210> SEQ ID NO 10
<211> LENGTH: 32
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: peptide

<400> SEQUENCE: 10

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Pro
1 5 10 15

Asn Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala
20 25 30

<210> SEQ ID NO 11
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: peptide
<223> OTHER INFORMATION: Cyclic peptide; Secondary structure is maintained by disulfide bond between intramolecular Cys residues at positions 9 and 31

-continued

<400> SEQUENCE: 11

Ile Glu Gly Pro Thr Leu Arg Gln Cys Leu Ala Ala Arg Ala Gly Gly
1 5 10 15

Gly Gly Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Cys Leu
20 25 30

Ala Ala Arg Ala
35

<210> SEQ ID NO 12
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: peptide

<400> SEQUENCE: 12

Ile Glu Gly Pro Thr Leu Arg Gln Cys Leu Ala Ala Arg Ala Gly Gly
1 5 10 15

Gly Gly Gly Gly Gly Gly Ile Glu Gly Pro Thr Arg Leu Gln Cys Leu
20 25 30

Ala Ala Arg Ala
35

<210> SEQ ID NO 13
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: peptide

<400> SEQUENCE: 13

Ile Glu Gly Pro Thr Leu Arg Gln Ala Leu Ala Ala Arg Ala Gly Gly
1 5 10 15

Gly Gly Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Ala Leu
20 25 30

Ala Ala Arg Ala
35

<210> SEQ ID NO 14
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: peptide

<400> SEQUENCE: 14

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Gly
1 5 10 15

Gly Lys Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu
20 25 30

Ala Ala Arg Ala
35

<210> SEQ ID NO 15
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Lys residue at position 18 is Bromoacetylated
<223> OTHER INFORMATION: Description of Artificial Sequence: derivatized peptide

-continued

<400> SEQUENCE: 15

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Gly
1 5 10 15

Gly Lys Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu
20 25 30

Ala Ala Arg Ala
35

<210> SEQ ID NO 16
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: peptide

<400> SEQUENCE: 16

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Gly
1 5 10 15

Gly Cys Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu
20 25 30

Ala Ala Arg Ala
35

<210> SEQ ID NO 17
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Lys at position 18 is pegylated
<223> OTHER INFORMATION: Description of Artificial Sequence: derivatized peptide

<400> SEQUENCE: 17

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Gly
1 5 10 15

Gly Lys Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu
20 25 30

Ala Ala Arg Ala
35

<210> SEQ ID NO 18
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Cys at position 18 is pegylated
<223> OTHER INFORMATION: Description of Artificial Sequence: derivatized peptide

<400> SEQUENCE: 18

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Gly
1 5 10 15

Gly Cys Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu
20 25 30

Ala Ala Arg Ala
35

<210> SEQ ID NO 19
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: peptide

-continued

<400> SEQUENCE: 19

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Gly
1 5 10 15

Gly Asn Gly Ser Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu
20 25 30

Ala Ala Arg Ala
35

<210> SEQ ID NO 20
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Monomeric subunit of a homodimer; Subunits in the homodimer are bonded by a disulfide bond between Cys residues at position 18 on each subunit
<223> OTHER INFORMATION: Description of Artificial Sequence: peptide

<400> SEQUENCE: 20

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Gly
1 5 10 15

Gly Cys Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu
20 25 30

Ala Ala Arg Ala
35

<210> SEQ ID NO 21
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: peptide

<400> SEQUENCE: 21

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Gly
1 5 10 15

Gly Gly Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu
20 25 30

Ala Ala Arg Ala
35

<210> SEQ ID NO 22
<211> LENGTH: 32
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Peptide is derivatized at the amino teminus with a covalently bonded immunoglobulin Fc region
<223> OTHER INFORMATION: Description of Artificial Sequence: peptide

<400> SEQUENCE: 22

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Pro
1 5 10 15

Asn Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala
20 25 30

<210> SEQ ID NO 23
<211> LENGTH: 32
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Peptide is covalently bonded at the animo and carboxy termini to an immunoglobulin Fc region

-continued

<223> OTHER INFORMATION: Description of Artificial Sequence: peptide

<400> SEQUENCE: 23

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Pro
1 5 10 15

Asn Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala
20 25 30

<210> SEQ ID NO 24
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Peptide is copvalently bonded at the carboxy terminus to an immunoglobulin Fc region
<223> OTHER INFORMATION: Description of Artificial Sequence: peptide

<400> SEQUENCE: 24

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Gly
1 5 10 15

Gly Gly Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu
20 25 30

Ala Ala Arg Ala
35

<210> SEQ ID NO 25
<211> LENGTH: 34
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Peptide is covalently bonded at the amino terminus to an immunoglobulin Fc region
<223> OTHER INFORMATION: Description of Artificial Sequence: peptide

<400> SEQUENCE: 25

Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala
1 5 10 15

Gly Pro Asn Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala
20 25 30

Arg Ala

<210> SEQ ID NO 26
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Peptide is covalently bonded at the amino terminus to an immunoglobulin Fc region
<223> OTHER INFORMATION: Description of Artificial Sequence: peptide

<400> SEQUENCE: 26

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Gly
1 5 10 15

Gly Gly Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu
20 25 30

Ala Ala Arg Ala
35

<210> SEQ ID NO 27
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Peptide is covalently bonded at the amino

-continued

terminus to an immunoglobulin Fc region
<223> OTHER INFORMATION: Cyclic peptide; Secondary structure is maintained by disulfide linkage between intramolecular Cys residues at positions 9 and 31
<223> OTHER INFORMATION: Description of Artificial Sequence: peptide

<400> SEQUENCE: 27

Ile Glu Gly Pro Thr Leu Arg Gln Cys Leu Ala Ala Arg Ala Gly Gly
1 5 10 15

Gly Gly Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Cys Leu
20 25 30

Ala Ala Arg Ala
35

<210> SEQ ID NO 28
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Peptide is covalently bonded at the amino terminus to an immunoglobulin Fc region
<223> OTHER INFORMATION: Description of Artificial Sequence: peptide

<400> SEQUENCE: 28

Ile Glu Gly Pro Thr Leu Arg Gln Cys Leu Ala Ala Arg Ala Gly Gly
1 5 10 15

Gly Gly Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Cys Leu
20 25 30

Ala Ala Arg Ala
35

<210> SEQ ID NO 29
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: peptide
<223> OTHER INFORMATION: Peptide is covalently bonded at the amino terminus to an immunoglobulin Fc region

<400> SEQUENCE: 29

Ile Glu Gly Pro Thr Leu Arg Gln Ala Leu Ala Ala Arg Ala Gly Gly
1 5 10 15

Gly Gly Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Ala Leu
20 25 30

Ala Ala Arg Ala
35

<210> SEQ ID NO 30
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Peptide is covalently bonded at the amino terminus to an immunoglobulin Fc region
<223> OTHER INFORMATION: Description of Artificial Sequence: peptide

<400> SEQUENCE: 30

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Gly
1 5 10 15

Gly Lys Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu
20 25 30

Ala Ala Arg Ala
35

-continued

<210> SEQ ID NO 31
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Peptide is covalently bonded at the amino terminus to an immunoglobulin Fc region
<223> OTHER INFORMATION: Description of Artificial Sequence: peptide

<400> SEQUENCE: 31

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Gly
1 5 10 15

Gly Cys Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu
20 25 30

Ala Ala Arg Ala
35

<210> SEQ ID NO 32
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: peptide
<223> OTHER INFORMATION: Peptide is covalently bonded at the amino terminus to an immunoglobulin Fc region

<400> SEQUENCE: 32

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Gly
1 5 10 15

Gly Asn Gly Ser Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu
20 25 30

Ala Ala Arg Ala
35

<210> SEQ ID NO 33
<211> LENGTH: 36
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: peptide
<223> OTHER INFORMATION: Peptide is a subunit of a homodimer; Subunits in the homodimer are colvantly bonded through a disulfide bond between Cys residues at position 18 of each subunit
<223> OTHER INFORMATION: Peptide is covalently bonded at the amino terminus to an immunoglobulin Fc region

<400> SEQUENCE: 33

Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Gly Gly
1 5 10 15

Gly Cys Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu
20 25 30

Ala Ala Arg Ala
35

<210> SEQ ID NO 34
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: peptide
<223> OTHER INFORMATION: Peptide is covalently bonded at the amino terminus to an immunoglobulin Fc region

<400> SEQUENCE: 34

-continued

Gly Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala
1 5 10 15

Ala Arg Ala Gly Gly Gly Gly Gly Gly Gly Ile Glu Gly Pro Thr
20 25 30

Leu Arg Gln Trp Leu Ala Ala Arg Ala
35 40

<210> SEQ ID NO 35
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: oligonucleotide

<400> SEQUENCE: 35

aaaggtggag gtggtggtat cgaaggtccg actctgcgtc agtggctggc tgctcgtgct 60

<210> SEQ ID NO 36
<211> LENGTH: 48
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: oligonucleotide

<400> SEQUENCE: 36

acctccacca ccagcacgag cagccagcca ctgacgcaga gtcggacc 48

<210> SEQ ID NO 37
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: oligonucleotide

<400> SEQUENCE: 37

ggtggtggag gtggcggcgg aggtattgag ggcccaaccc ttcgccaatg gcttgcagca 60

cgcgca 66

<210> SEQ ID NO 38
<211> LENGTH: 76
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: oligonucleotide

<400> SEQUENCE: 38

aaaaaaagga tcctcgagat tatgcgcgtg ctgcaagcca ttggcgaagg gttgggccct 60

caatacctcc gccgcc 76

<210> SEQ ID NO 39
<211> LENGTH: 126
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: oligonucleotide

<400> SEQUENCE: 39

aaaggtggag gtggtggtat cgaaggtccg actctgcgtc agtggctggc tgctcgtgct 60

ggtggtggag gtggcggcgg aggtattgag ggcccaaccc ttcgccaatg gcttgcagca 120

-continued

cgcgca 126

<210> SEQ ID NO 40
<211> LENGTH: 124
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: oligonucleotide

<400> SEQUENCE: 40

ccaggctgag acgcagtcac cgaccgacga gcacgaccac cacctccacc gccgcctcca 60

taactcccgg gttgggaagc ggttaccgaa cgtcgtgcgc gtattagagc tcctaggaaa 120

aaaa 124

<210> SEQ ID NO 41
<211> LENGTH: 42
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: peptide

<400> SEQUENCE: 41

Lys Gly Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu
1 5 10 15

Ala Ala Arg Ala Gly Gly Gly Gly Gly Gly Gly Gly Ile Glu Gly Pro
20 25 30

Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala
35 40

<210> SEQ ID NO 42
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: oligonucleotide

<400> SEQUENCE: 42

aacataagta cctgtaggat cg 22

<210> SEQ ID NO 43
<211> LENGTH: 52
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: oligonucleotide

<400> SEQUENCE: 43

ttcgatacca ccacctccac ctttacccgg agacagggag aggctcttct gc 52

<210> SEQ ID NO 44
<211> LENGTH: 861
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: oligonucleotide

<400> SEQUENCE: 44

tctagatttg ttttaactaa ttaaaggagg aataacatat ggacaaaact cacacatgtc 60

caccttgtcc agctccggaa ctcctggggg gaccgtcagt cttcctcttc cccccaaaac 120

-continued

ccaaggacac cctcatgatc tcccggaccc ctgaggtcac atgcgtggtg gtggacgtga 180

gccacgaaga ccctgaggtc aagttcaact ggtacgtgga cggcgtggag gtgcataatg 240

ccaagacaaa gccgcgggag gagcagtaca acagcacgta ccgtgtggtc agcgtcctca 300

ccgtcctgca ccaggactgg ctgaatggca aggagtacaa gtgcaaggtc tccaacaaag 360

ccctcccagc ccccatcgag aaaaccatct ccaaagccaa agggcagccc cgagaaccac 420

aggtgtacac cctgccccca tcccgggatg agctgaccaa gaaccaggtc agcctgacct 480

gcctggtcaa aggcttctat cccagcgaca tcgccgtgga gtgggagagc aatgggcagc 540

cggagaacaa ctacaagacc acgcctcccg tgctggactc cgacggctcc ttcttcctct 600

acagcaagct caccgtggac aagagcaggt ggcagcaggg gaacgtcttc tcatgctccg 660

tgatgcatga ggctctgcac aaccactaca cgcagaagag cctctccctg tctccgggta 720

aaggtggagg tggtggtatc gaaggtccga ctctgcgtca gtggctggct gctcgtgctg 780

gtggtggagg tggcggcgga ggtattgagg gcccaaccct tcgccaatgg cttgcagcac 840

gcgcataatc tcgaggatcc g 861

<210> SEQ ID NO 45
<211> LENGTH: 861
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
oligonucleotide

<400> SEQUENCE: 45

agatctaaac aaaattgatt aatttcctcc ttattgtata cctgttttga gtgtgtacag 60

gtggaacagg tcgaggcctt gaggaccccc ctggcagtca gaaggagaag gggggttttg 120

ggttcctgtg ggagtactag agggcctggg gactccagtg tacgcaccac cacctgcact 180

cggtgcttct gggactccag ttcaagttga ccatgcacct gccgcacctc cacgtattac 240

ggttctgttt cggcgccctc ctcgtcatgt tgtcgtgcat ggcacaccag tcgcaggagt 300

ggcaggacgt ggtcctgacc gacttaccgt tcctcatgtt cacgttccag aggttgtttc 360

gggagggtcg ggggtagctc ttttggtaga ggtttcggtt tcccgtcggg gctcttggtg 420

tccacatgtg ggacgggggt agggccctac tcgactggtt cttggtccag tcggactgga 480

cggaccagtt tccgaagata gggtcgctgt agcggcacct caccctctcg ttacccgtcg 540

gcctcttgtt gatgttctgg tgcggagggc acgacctgag gctgccgagg aagaaggaga 600

tgtcgttcga gtggcacctg ttctcgtcca ccgtcgtccc cttgcagaag agtacgaggc 660

actacgtact ccgagacgtg ttggtgatgt gcgtcttctc ggagagggac agaggcccat 720

ttccacctcc accaccatag cttccaggct gagacgcagt caccgaccga cgagcacgac 780

caccacctcc accgccgcct ccataactcc cgggttggga agcggttacc gaacgtcgtg 840

cgcgtattag agctcctagg c 861

<210> SEQ ID NO 46
<211> LENGTH: 269
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: peptide

<400> SEQUENCE: 46

Met Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu

-continued

```
 1               5                  10                  15

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
            20                  25                  30

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
        35                  40                  45

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
    50                  55                  60

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr
65                  70                  75                  80

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
                85                  90                  95

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro
            100                 105                 110

Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
        115                 120                 125

Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val
    130                 135                 140

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
145                 150                 155                 160

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
                165                 170                 175

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
            180                 185                 190

Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
        195                 200                 205

Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
    210                 215                 220

Ser Pro Gly Lys Gly Gly Gly Gly Gly Ile Glu Gly Pro Thr Leu Arg
225                 230                 235                 240

Gln Trp Leu Ala Ala Arg Ala Gly Gly Gly Gly Gly Gly Gly Gly Ile
                245                 250                 255

Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala
            260                 265
```

What is claimed is:

1. A compound that binds to an Mp1 receptor comprising the structure

$$TMP_1\text{-}(L_1)_n\text{-}TMP_2$$

wherein TMP_1, and TMP_2 are each Ile-Glu-Gly-Pro-Thr-Leu-Arg-Gln-Trp-Leu-Ala-Ala-Arg-Ala. (SEQ ID NO: 1);

L_1 is a linker; and

n is 0 or 1;

and physiologically acceptable salts thereof.

2. A compound that binds to an Mp1 receptor, which is selected from the group consisting of

(SEQ ID NO: 10)

IEGPTLRQWLAARA-GPNG-IEGPTLRQWLAARA

(SEQ ID NO: 11)

IEGPTLRQCLAARA-GGGGGGGG-IEGPTLRQCLAARA (cyclic)

-continued

(SEQ ID NO: 12)

IEGPTLRQCLAARA-GGGGGGGG-IEGPTLRQCLAARA (linear)

(SEQ ID NO: 13)

IEGPTLRQALAARA-GGGGGGGG-IEGPTLRQALAARA

(SEQ ID NO: 14)

IEGPTLRQWLAARA-GGGKGGGG-IEGPTLRQWLAARA

(SEQ ID NO: 15)

IEGPTLRQWLAARA-GGGK(BrAc)GGGG-IEGPTLRQWLAARA

(SEQ ID NO: 16)

IEGPTLRQWLAARA-GGGCGGGG-IEGPTLRQWLAARA

(SEQ ID NO: 17)

IEGPTLRQWLAARA-GGGK(PEG)GGGG-IEGPTLRQWLAARA

(SEQ ID NO: 18)

IEGPTLRQWLAARA-GGGC(PEG)GGGG-IEGPTLRQWLAARA

(SEQ ID NO: 19)

IEGPTLRQWLAARA-GGGNGSGG-IEGPTLRQWLAARA

-continued

(SEQ ID NO: 20)

IEGPTLRQWLAARA-GGGCGGGG-IEGPTLRQWLAARA

IEGPTLRQWLAARA-GGGCGGGG-IEGPTLRQWLAARA;

(SEQ ID NO: 21)

IEGPTLRQWLAARA-GGGGGGGG-IEGPTLRQWLAARA.

3. A compound that binds to an Mpl receptor, which is selected from the group consisting of

(SEQ ID NO: 22)

Fc-IEGPTLRQWLAARA-GPNG-IEGPTLRQWLAARA

(SEQ ID NO: 23)

Fc-IEGPTLRQWLAARA-GPNG-IEGPTLRQWLAARA-Fc

(SEQ ID NO: 24)

IEGPTLRQWLAARA-GGGGGGGG-IEGPTLRQWLAARA-Fc

(SEQ ID NO: 25)

Fc-FF-IEGPTLRQWLAARA-GPNG-IEGPTLRQWLAARA

(SEQ ID NO: 26)

Fc-IEGPTLRQWLAARA-GGGGGGGG-IEGPTLRQWLAARA

-continued

(SEQ ID NO: 27)

Fc-IEGPTLRQCLAARA-GGGGGGGG-IEGPTLRQCLAARA (cyclic)

(SEQ ID NO: 28)

Fc-IEGPTLRQCLAARA-GGGGGGGG-IEGPTLRQCLAARA (linear)

(SEQ ID NO: 29)

Fc-IEGPTLRQALAARA-GGGGGGGG-IEGPTLRQALAARA

(SEQ ID NO: 30)

Fc-IEGPTLRQWLAARA-GGGKGGGG-IEGPTLRQWLAARA

(SEQ ID NO: 31)

Fc-IEGPTLRQWLAARA-GGGCGGGG-IEGPTLRQWLAARA

(SEQ ID NO: 32)

Fc-IEGPTLRQWLAARA-GGGNGSGG-IEGPTLRQWLAARA

(SEQ ID NO: 33)

Fc-IEGPTLRQWLAARA-GGGCGGGG-IEGPTLRQWLAARA

Fc-IEGPTLRQWLAARA-GGGCGGGG-IEGPTLRQWLAARA

(SEQ ID NO: 34)

Fc-GGGGG-IEGPTLRQWLAARA-GGGGGGGG-IEGPTLRQWLAARA

and physiologically acceptable salts thereof.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,835,809 B1
DATED : October 22, 1999
INVENTOR(S) : Liu et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 65,
Line 21, please delete "Fc-FF-IEG" and insert -- Fc-GG-IEG --.

Signed and Sealed this

Twenty-fourth Day of May, 2005

JON W. DUDAS
Director of the United States Patent and Trademark Office

EXHIBIT B

Maintenance fee payment

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450
www.uspto.gov

Customer No 000000 LKBOX DATE PRINTED 06/30/2008

MARSHALL O'TOOLE GERSTEIN
MURRAY & BORUN
6300 SEARS TOWER
233 S WACKER DRIVE
CHICAGO IL 60606-6402

MAINTENANCE FEE STATEMENT

According to the records of the U.S. Patent and Trademark Office (USPTO), the maintenance fee and any necessary surcharge have been timely paid for the patent listed below. The "PYMT DATE" column indicates the payment date (i.e., the date the payment was filed).

The payment shown below is subject to actual collection. If the payment is refused or charged back by a financial institution, the payment will be void and the maintenance fee and any necessary surcharge unpaid.

Direct any questions about this statement to: Mail Stop M Correspondence, Director of the USPTO, P.O. Box 1450, Alexandria, VA 22313-1450.

PATENT NUMBER	FEE AMT	SUR CHARGE	PYMT DATE	U.S. APPLICATION NUMBER	PATENT ISSUE DATE	APPL. FILING DATE	PAYMENT YEAR	SMALL ENTITY?	ATTY DKT NUMBER
6,835,809	930	0	06/13/08	09/422,838	12/28/04	10/22/99	04	NO	01017/36263

EXHIBIT C

Copy of Request for Certificate of Correction

I hereby certify that this correspondence is being deposited with the U.S. Postal Service with sufficient postage as First Class Mail, in an envelope addressed to: MS Post Issue, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on the date shown below.

Dated: January 31, 2005 Signature: Joseph A. Williams, Jr.

Docket No.: 01017/36263
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Letters Patent of:
Liu et al.

Patent No.: 6,835,809 B1

Issued: December 28, 2004

For: Thrombopoietic Compounds

REQUEST FOR CERTIFICATE OF CORRECTION
PURSUANT TO 37 CFR 1.323

MS Post Issue
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Upon reviewing the above-identified patent, Patentee noted typographical errors which should be corrected.

Appln. Page #	Appln. Line #	Column #	Line#	Error By
Amendment filed 04-12-2004		65	21	PTO

The error was not in the application as filed by applicant or any other papers filed by applicant. Accordingly, no fee is required.

Transmitted herewith is a proposed Certificate of Correction effecting such amendment. Patentee respectfully solicits the granting of the requested Certificate of Correction.

The Director is hereby authorized to charge any deficiency in the fees filed to our Deposit Account No. 13-2855. A duplicate copy of this paper is enclosed.

Dated: January 31, 2005

Respectfully submitted,

By _____
Joseph A. Williams, Jr.
Registration No.: 38,659
MARSHALL, GERSTEIN & BORUN LLP
233 S. Wacker Drive, Suite 6300
Sears Tower
Chicago, Illinois 60606-6357
(312) 474-6300
Attorney for Applicant

FEB 14 2005

PTO/SB/44 (04-04)
Approved for use through 04/30/2007. OMB 0651-0033
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

(Also Form PTO-1050)

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 6,835,809 B1

DATED : October 22, 1999

INVENTOR(S) : Liu et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Col. 65, line 21, Please delete "Fc-FF-IEG" and insert --Fc-GG-IEG--.

MAILING ADDRESS OF SENDER:
Joseph A. Williams, Jr.
MARSHALL, GERSTEIN & BORUN LLP
233 S. Wacker Drive, Suite 6300
Sears Tower
Chicago, Illinois 60606-6357

PATENT NO. 6,835,809

No. of additional copies 0

FEB 14 2005

EXHIBIT D

FDA letter acknowledging receipt of IND

DEPARTMENT OF HEALTH & HUMAN SERVICES

Food and Drug Administration
1401 Rockville Pike
Rockville MD 20852-1448

JAN 16 2002

Our Reference: BB-IND 10205

Amgen, Incorporated
Attention: Mr. Doug Hunt
Manager, Regulatory Affairs
One Amgen Center Drive
Thousand Oaks, CA 91320

Dear Mr. Hunt:

The Center for Biologics Evaluation and Research has received your **Investigational New Drug Application (IND)**. The following product name and BB-IND number have been assigned to this application. They serve only to identify it and do not imply that this Center either endorses or does not endorse your application.

BB-IND #: 10205

SPONSOR: Amgen, Incorporated

PRODUCT NAME: Thrombopoietin Analogue:Fc Fusion Protein (AMG 531) to the Thrombopoietin Receptor

DATE OF SUBMISSION: December 20, 2001

DATE OF RECEIPT: December 28, 2001

This BB-IND number should be used to identify all future correspondence and submissions, as well as telephone inquiries concerning this IND. Please provide an **original and two copies of every submission to this file**. Please include three originals of all illustrations which do not reproduce well.

It is understood that studies in humans will not be initiated until 30 days after the date of receipt shown above. If this office notifies you, verbally or in writing, of serious deficiencies that require correction before human studies can begin, it is understood that you will continue to withhold such studies until you are notified that the material you have submitted to correct the deficiencies is satisfactory. If such a clinical hold is placed on this file, you will be notified in writing of the reasons for placing the IND on hold.

You are responsible for compliance with applicable portions of the Public Health Service Act, the Federal Food, Drug, and Cosmetic Act, and the Code of Federal Regulations (CFR). A copy of 21 CFR Part 312, pertaining to INDs, is enclosed. Copies of other pertinent regulations are available from this Center upon request. The following points regarding obligations of an IND sponsor are included for your information only, and are not intended to be comprehensive.

Progress reports are required at intervals not exceeding one year and are due within 60 days of the anniversary of the date that the IND went into effect [21 CFR 312.33]. Any unexpected, fatal or immediately life-threatening reaction associated with use of this product must be reported to this Division by telephone or facsimile transmission no later than seven calendar days after initial receipt of the information. All serious, unexpected adverse experiences, as well as results from animal studies that suggest significant clinical risk, must be reported, in writing, to this Division and to all investigators within fifteen calendar days after initial receipt of this information [21 CFR 312.32].

Charging for an investigational product in a clinical trial under an IND is not permitted without the prior written approval of the FDA.

Prior to use of each new lot of the investigational biologic in clinical trials, please submit the lot number, the results of all tests performed on the lot, and the specifications when established (i.e., the range of acceptable results).

If not included in your submission, please provide copies of the consent forms for each clinical study. A copy of the requirements for and elements of informed consent are enclosed. Also, please provide documentation of the institutional review board approval(s) for each clinical study.

All laboratory or animal studies intended to support the safety of this product should be conducted in compliance with the regulations for "Good Laboratory Practice for Nonclinical Laboratory Studies" (21 CFR Part 58, copies available upon request). If such studies have not been conducted in compliance with these regulations, please provide a statement describing in detail all differences between the practices used and those required in the regulations.

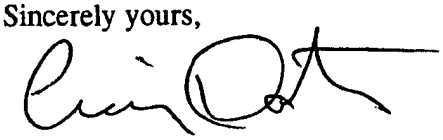
Item 7a of form FDA 1571 requests that either an "environmental assessment," or a "claim for categorical exclusion" from the requirements for environmental assessment, be included in the IND. If you did not include a response to this item with your application, please submit one. See the enclosed information sheet for additional information on how these requirements may be addressed.

Telephone inquiries concerning this IND should be made directly to me at (301) 827-5101. Correspondence regarding this file should be addressed as follows:

Center for Biologics Evaluation and Research
Attn: Office of Therapeutics Research and Review
HFM-99, Room 200N
1401 Rockville Pike
Rockville, MD 20852-1448

If we have any comments after we have reviewed this submission, we will contact you.

Sincerely yours,



Craig Doty, Pharm.D.
Regulatory Project Manager
Division of Application Review and Policy
Office of Therapeutics
Research and Review
Center for Biologics
Evaluation and Research

Enclosures (3): 21 CFR Part 312
21 CFR 50.20, 50.25
Information sheet on 21 CFR 25.24

EXHIBIT E

FDA letter acknowledging effective date of IND

DEPARTMENT OF HEALTH & HUMAN SERVICES

Food and Drug Administration
1401 Rockville Pike
Rockville MD 20852-1448

APR 19 2002

Our Reference: BB-IND 10205

Amgen, Incorporated
Attention: Douglas Hunt
Manager, Regulatory Affairs
One Amgen Center Drive
Thousand Oaks, CA 91320

Dear Mr. Hunt:

We have reviewed the March 20, 2002, submission to your **Investigational New Drug Application (IND)** for "Thrombopoietin Analogue:Fc Fusion Protein (AMG 531) (Amgen) to the Thrombopoietin Receptor (c-Mpl)." We also acknowledge receipt of your April 11, 2002, facsimile transmission which should be submitted officially as an amendment to your IND.

You have satisfactorily addressed the issues raised in our letter of February 21, 2002. The clinical hold has been removed and your proposed study may proceed contingent upon completing and submitting a revised protocol incorporating the following changes, as agreed to during the April 11, 2002, telephone conversation between you and Dr. Scott Proestel of this office:

1. The component of the study's stopping rules relating to the detection of thrombopoietin neutralizing antibodies will be revised to require that subject enrollment cease following the detection of neutralizing antibodies to thrombopoietin within any subject. This revision will delete any requisite clinical correlates of the neutralizing antibodies.

2. The protocol will be revised to specifically identify all the blood antibody tests performed during follow-up clinical evaluations.

You do not have to wait for FDA review of the above submitted information and authorization to proceed before initiating your proposed study.

You are responsible for compliance with the Federal Food, Drug, and Cosmetic Act, and the Code of Federal Regulations (CFR). Progress reports are required at intervals not exceeding one year and are due within 60 days of the anniversary of the date that the IND went into effect [21 CFR 312.33]. Any unexpected, fatal or immediately life-threatening reaction associated with use of this product must be reported to this Division by telephone or facsimile transmission no later than seven calendar days after initial receipt of the information. All serious, unexpected adverse experiences, as well as results from animal studies that suggest significant clinical risk, must be reported, in writing, to this Division and to all investigators within fifteen calendar days after initial receipt of this information [21 CFR 312.32].

If you have any questions, please contact the Regulatory Project Manager, Dr. Craig Doty, at (301) 827-5101.

Sincerely yours,

Carl Dyz

for Glen D. Jones, Ph.D.
Director
Division of Application Review and Policy
Office of Therapeutics
Research and Review
Center for Biologics
Evaluation and Research

EXHIBIT F
FDA letter approving BLA No. 125268/0

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Rockville, MD 20857

Our STN: BL 125268/0

AUG 22 2008

Amgen, Inc.
ATTENTION: Mei-Ling Chang-Lok, Ph.D., RAC
Director, Regulatory Affairs
One Amgen Center Drive
Thousand Oaks, CA 91320-1799

Dear Dr. Chang-Lok:

We have approved your biologics license application for romiplostim effective this date. You are hereby authorized to introduce or deliver for introduction into interstate commerce, romiplostim under your existing Department of Health and Human Services U.S. License No. 1080. Romiplostim is indicated for the treatment of thrombocytopenia in patients with chronic immune (idiopathic) thrombocytopenic purpura (ITP) who have had an insufficient response to corticosteroids, immunoglobulins, or splenectomy.

Under this license, you are approved to manufacture romiplostim drug substance at Amgen Inc, in Boulder, Colorado. The final formulated product will be manufactured, filled, labeled, and packaged at Amgen, Inc, Patheon Italia, Monza, Italy. You may label your product with the proprietary name, Nplate™, and may market it in 250 mcg and 500 mcg vials.

The final printed labeling (FPL) must be identical to the enclosed labeling. Marketing product with FPL that is not identical to the approved labeling text may render the product misbranded and an unapproved new drug.

The dating period for romiplostim shall be from the date of manufacture when stored at . The date of manufacture shall be defined as the date of final of the formulated drug product. The dating period for your drug substance shall be 36 months when stored at -30 °C. We have approved the stability protocol(s) in your license application for the purpose of extending the expiration dating period of your drug substance and drug product under 21 CFR 601.12.

You currently are not required to submit samples of future lots of romiplostim to the Center for Drug Evaluation and Research (CDER) for release by the Director, CDER, under 21 CFR 610.2. We will continue to monitor compliance with 21 CFR 610.1 requiring completion of tests for conformity with standards applicable to each product prior to release of each lot.

You must submit information to your biologics license application for our review and written approval under 21 CFR 601.12 for any changes in the manufacturing, testing, packaging or labeling of Romiplostim, or in the manufacturing facilities.

REQUIRED PEDIATRIC ASSESSMENTS

Under the Pediatric Research Equity Act (PREA) (21 U.S.C. 355c), all applications for new active ingredients, new indications, new dosage forms, new dosing regimens, or new routes of administration are required to contain an assessment of the safety and effectiveness of the product for the claimed indications in pediatric patients unless this requirement is waived, deferred, or inapplicable.

Because this biological product for this indication has an orphan drug designation, you are exempt from this requirement.

POSTMARKETING REQUIREMENTS UNDER 505(o)

Title IX, Subtitle A, Section 901 of the Food and Drug Administration Amendments Act of 2007 (FDAAA) amends the Federal Food, Drug, and Cosmetic Act (FDCA) to authorize FDA to require holders of approved drug and biological product applications to conduct postmarketing studies and clinical trials for certain purposes, if FDA makes certain findings required by the statute (section 505(o)(3)(A), 21 U.S.C. 355(o)(3)(A)). This provision took effect on March 25, 2008.

We have determined that an analysis of spontaneous postmarketing adverse events reported under subsection 505(k)(1) will not be sufficient to assess the signals of the following serious risks in patients with chronic ITP who are receiving romiplostim: bone marrow reticulin formation and a risk for bone marrow fibrosis; antibody formation to either romiplostim or thrombopoietin that results in worsened thrombocytopenia; off-target cardiac toxicities; or to identify unexpected serious risks of adverse reactions within the fetus of pregnant woman and in the nursing infants of women who are receiving romiplostim.

Furthermore, the new pharmacovigilance system that FDA is required to establish under section 505(k)(3) has not yet been established and is therefore not sufficient to assess these signals of serious risks or to identify unexpected serious risks.

Therefore, based on appropriate scientific data, FDA has determined that you are required, pursuant to section 505(o)(3) of the FDCA, to conduct the following studies.

1. To conduct an "Antibody Registry Study" that will enroll subjects who have received romiplostim and whose blood samples contain antibodies to either romiplostim or thrombopoietin. The antibody assays will be performed by Amgen in response to spontaneously submitted requests for the post-marketing blood tests. As described in the romiplostim prescribing information, a lack or loss of response to romiplostim should prompt the healthcare provider to search for causative factors, including neutralizing antibodies to romiplostim. In these situations, healthcare providers are to submit blood samples to Amgen for detection of antibodies to romiplostim and thrombopoietin.

The Antibody Registry Study will collect follow-up platelet count and other clinical data sufficient to assess the long term consequences of the detected antibodies. Patients will be followed until the detected antibodies resolve or stabilize in titer over a several month period of time.

You will conduct this study according to the following timetable:

Protocol Submission:	November 2008
Study Start:	May 2009
First interim report submission:	May 2010 then annually
Final Report Submission:	Within six months of FDA notification that sufficient data has been collected

2. To develop and maintain a prospective, observational pregnancy exposure registry study conducted in the United States that compares the pregnancy and fetal outcomes of women exposed to romiplostim during pregnancy to an unexposed control population. The registry will detect and record major and minor congenital anomalies, spontaneous abortions, stillbirths, elective terminations, adverse effects on immune system development, platelet number and function, neoplasm formation, bone marrow reticulin formation, thrombotic events, and any serious adverse pregnancy outcomes. These events will be assessed among the enrolled women throughout the pregnancy. The events will also be assessed among infants through at least the first year of life. Annual interim reports will be submitted until FDA has acknowledged that sufficient data have been collected.

   You will conduct this study according to the following timetable:

Protocol Submission:	November 2008
Study Start:	May 2009
First interim report submission:	May 2010 then annually
Final Report:	Within six months of FDA notification that sufficient data has been collected.

3. To conduct a milk only lactation study in the subset of women enrolled in the pregnancy registry that choose to breastfeed their infants. This study will be designed to detect the presence and concentration of romiplostim in breast milk and any effects on milk production and composition. The study will include a symptom diary for mothers to record any adverse effects in the breastfeeding infants. Annual interim reports will be submitted until FDA has acknowledged that sufficient data have been collected.

   You will conduct this study according to the following timetable:

Protocol Submission:	November 2008
Study Start:	May 2009
First interim report submission:	May 2010 then annually
Final Report:	Within six months of FDA notification that sufficient data has been collected.

Finally, we have determined that only a clinical trial (rather than a nonclinical or observational study) in which patients with defined underlying risks are carefully evaluated for at least 24 hours following administration of romiplostim will be sufficient to assess the signals of serious risk or identify unexpected serious risks.

Therefore, based on appropriate scientific data, FDA has determined that you are required, pursuant to section 505(o)(3) of the FDCA, to conduct the following clinical trial.

4. To conduct trial 20080009, "A Prospective Phase IV, Open-Label, Multi-Center, Study Evaluating the Changes in Bone Marrow Morphology in Subjects Receiving Romiplostim for the Treatment of Thrombocytopenia associated with Immune (Idiopathic) Thrombocytopenia Purpura (ITP)." In this trial, at least 150 patients will receive romiplostim and undergo bone marrow evaluations prior to, during and following the completion of romiplostim administration. A similar evaluation schedule will apply to the detection of antibody formation to romiplostim and thrombopoietin as well as the electrocardiographic (ECG) detection of cardiac conduction abnormalities.

   A first interim report will contain, in addition to any other items, ECG and the results of bone marrow evaluations for patients who have completed 12 months of trial participation. This information will be updated for patients who have completed 24 months of trial participation and submitted in a second interim report.

   You will conduct this trial according to the following timetable:

Protocol submission:	August 22, 2008
Trial start:	July 2009
First interim report submission:	June 2012
Second interim report submission:	June 2013
Final report submission:	December 2014

Submit the protocols to your IND 10205 with a cross-reference letter to this BLA, STN BL 125268/0. Submit nonclinical and chemistry, manufacturing, and controls protocols and all study final reports to your BLA, STN BL 125268/0. Use the following designators to prominently label all submissions, including supplements, relating to these postmarketing studies and clinical trial as appropriate:

- **Required Postmarketing Protocol under 505(o)**
- **Required Postmarketing Final Report under 505(o)**
- **Required Postmarketing Correspondence under 505(o)**

You are required to report periodically to FDA on the status of these studies and clinical trial pursuant to sections 505(o)(3)(E)(ii) and 506B of the FDCA, as well as 21 CFR 601.70. Under section 505(o)(3)(E)(ii), you are also required to periodically report to FDA on the status of any study or trial otherwise undertaken to investigate a safety issue associated with romiplostim.

RISK EVALUATION AND MITIGATION STRATEGIES (REMS) REQUIREMENTS

Title IX, Subtitle A, Section 901 of FDAAA amends the FDCA to authorize FDA to require the submission of a Risk Evaluation and Mitigation Strategy (REMS) if FDA determines that such a strategy is necessary to ensure that the benefits of the drug outweigh the risks (section 505-1(a)). This provision took effect on March 25, 2008.

In accordance with section 505-1 of the FDCA, we have determined that a REMS is necessary for Nplate (romiplostim) Subcutaneous Injection to ensure the benefits of the drug outweigh the risks of bone marrow fibrosis, worsened thrombocytopenia after cessation of Nplate, thromboembolic complications, an increased risk of hematological malignancies and progression of malignancy in patients with a pre-existing hematological malignancy or myelodysplastic syndrome (MDS), and serious complications due to medication error. Pursuant to 505-1(f)(1), we have also determined that Nplate can be approved only if elements necessary to assure safe use are required as part of a REMS to mitigate these risks listed in the labeling.

Your proposed REMS, appended to this letter, submitted on August 12, 2008, in response to our July 25, 2008, information request letter, is approved. The REMS consists of a Medication Guide, a communication plan, elements to assure safe use, an implementation system, and a timetable for assessments of the REMS.

Prominently identify the amendment containing the REMS assessments or proposed modifications with the following wording in bold capital letters at the top of the first page of the submission:

- **BLA 125268 REMS ASSESSEMENT**
- **NEW SUPPLEMENT FOR BLA 125268 REMS ASSESSMENT PROPOSED REMS MODIFICATION**

Please note that:

- This Medication Guide must be reprinted immediately following the last section of labeling or, alternatively, accompany the prescription drug labeling [21 CFR 201.57(c)(18)] or 21 CFR 201.80(f)(2)];
- You are responsible for ensuring that this Medication Guide is available for distribution to every patient who is dispensed a prescription for this product [21 CFR 208.24];
- The final printed Medication Guide distributed to patients must conform to all conditions described in 21 CFR 208.20, including a minimum of 10 point text; and
- You are responsible for ensuring that the label of each container or package includes a prominent and conspicuous instruction to authorized dispensers to provide a Medication Guide to each patient to whom the drug is dispensed, and states how the Medication Guide is provided [21 CFR 208.24(d)].

CONTENT OF LABELING

Within 21 days of the date of this letter, submit content of labeling [21 CFR 601.14(b)] in structured product labeling (SPL) format, as described at http://www.fda.gov/oc/datacouncil/spl.html, that is identical in content to the enclosed labeling text. Upon receipt, we will transmit that version to the National Library of Medicine for public dissemination. For administrative purposes, please designate this submission "Product Correspondence – Final SPL for approved STN BL 125268/0." In addition, within 21 days of the date of this letter, amend any pending supplement(s) for this BLA with content of labeling in SPL format to include the changes approved in this supplement.

CARTON AND IMMEDIATE CONTAINER LABELS

Submit final printed carton and container labels that are identical to the enclosed draft labels as soon as they are available but no more than 30 days after they are printed. Please submit these labels electronically according to the guidance for industry titled *Providing Regulatory Submissions in Electronic Format – Human Pharmaceutical Product Applications and Related Submissions Using the eCTD Specifications (October 2005).* Alternatively, you may submit 12 paper copies, with 6 of the copies individually mounted on heavy-weight paper or similar material. For administrative purposes, designate this submission "**Product Correspondence – Final Printed Carton and Container Labels for approved STN BL 125268/0.**" Approval of this submission by FDA is not required before the labeling is used.

Marketing the product with labeling that is not identical to the approved labeling text may render the product misbranded and an unapproved new drug.

PROMOTIONAL MATERIALS

You may submit draft copies of the proposed introductory advertising and promotional labeling with a cover letter requesting advisory comments to the Food and Drug Administration, Center for Drug Evaluation and Research, Division of Drug Marketing, Advertising and Communication, 5901-B Ammendale Road, Beltsville, MD 20705-1266. Final printed advertising and promotional labeling should be submitted at the time of initial dissemination, accompanied by a FDA Form 2253.

All promotional claims must be consistent with and not contrary to approved labeling. You should not make a comparative promotional claim or claim of superiority over other products unless you have substantial evidence to support that claim.

REPORTING REQUIREMENTS

We remind you that you must comply with reporting requirements for an approved BLA (21 CFR 600.80).

We acknowledge your May 30, 2008, commitment to expedited reporting of bone marrow fibrosis, malignancy/malignancy progression, and medication error resulting in a serious adverse event.

Please refer to http://www.fda.gov/cder/biologics/default.htm for information regarding therapeutic biological products, including the addresses for submissions.

If you have any questions, please contact Florence Moore, M.S., Regulatory Project Manager, at (301) 796-2050.

Sincerely,

Richard Pazdur MD

Richard Pazdur, M.D.
Director
Office of Oncology Drug Products
Center for Drug Evaluation and Research

Enclosure: Package Insert
Carton and Vial Labeling
Medication Guide
Risk Mitigation and Evaluation Strategies (REMS)

EXHIBIT G

FDA-approved product package insert

HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use Nplate safely and effectively. See full prescribing information for Nplate.

Nplate™ (romiplostim)

For subcutaneous injection
Initial U.S. Approval: 2008

----------INDICATIONS AND USAGE----------

Nplate is a thrombopoietin receptor agonist indicated for the treatment of thrombocytopenia in patients with chronic immune (idiopathic) thrombocytopenic purpura (ITP) who have had an insufficient response to corticosteroids, immunoglobulins, or splenectomy.

Nplate should be used only in patients with ITP whose degree of thrombocytopenia and clinical condition increase the risk for bleeding. Nplate should not be used in an attempt to normalize platelet counts. (1)

----------DOSAGE AND ADMINISTRATION----------

- Initial dose of 1 mcg/kg once weekly as a subcutaneous injection. (2.1)
- Adjust weekly dose by increments of 1 mcg/kg to achieve and maintain a platelet count ≥ 50 x 10^9/L as necessary to reduce the risk for bleeding. (2.1)
- Do not exceed the maximum weekly dose of 10 mcg/kg. Do not dose if platelet count is > 400 x 10^9/L. (2.1)
- Discontinue Nplate if platelet count does not increase after 4 weeks at the maximum dose. (2.1)
- Do not shake during reconstitution; protect reconstituted Nplate from light; administer reconstituted Nplate within 24 hours. (2.2)
- The injection volume may be very small. Use a syringe with graduations to 0.01 mL. (2.2)
- Discard any unused portion of the single-use vial. (2.2)

----------DOSAGE FORMS AND STRENGTHS----------

- 250 mcg or 500 mcg of deliverable romiplostim in single-use vials (3)

----------CONTRAINDICATIONS ----------

- None (4)

----------WARNINGS AND PRECAUTIONS----------

- Nplate increases the risk for reticulin deposition within the bone marrow; clinical studies have not ruled out the possibility that reticulin and other fiber deposition may result in bone marrow fibrosis with cytopenias. Monitor peripheral blood for signs of marrow fibrosis. (5.1)
- Discontinuation of Nplate may result in worsened thrombocytopenia than was present prior to Nplate therapy. Monitor complete blood counts (CBCs), including platelet counts, for at least 2 weeks following Nplate discontinuation. (5.2)
- Excessive Nplate doses may increase platelet counts to a level that produces thrombotic/thromboembolic complications. (5.3)
- Assess patients for the formation of neutralizing antibodies if platelet counts importantly decrease following an initial Nplate response. (5.4)
- Nplate may increase the risk for hematological malignancies, especially in patients with myelodysplastic syndrome. (5.5)
- Monitor CBCs, including platelet counts and peripheral blood smears, weekly until a stable Nplate dose has been achieved. Thereafter, monitor CBCs, including platelet counts and peripheral blood smears, at least monthly. (5.6)
- Nplate is available only through a restricted distribution program called the Nplate NEXUS (Network of Experts Understanding and Supporting Nplate and Patients) Program. Under the Nplate NEXUS Program, only prescribers and patients registered with the program are able to prescribe, administer, and receive product. To enroll in the Nplate NEXUS Program, call 1-877-Nplate1 (1-877-675-2831). (5.7)

----------ADVERSE REACTIONS----------

The most common adverse reactions (≥ 5% higher patient incidence in Nplate versus placebo) are arthralgia, dizziness, insomnia, myalgia, pain in extremity, abdominal pain, shoulder pain, dyspepsia, and paresthesia. Headache was the most commonly reported adverse reaction that did not occur at ≥ 5% higher patient incidence in Nplate versus placebo. (6.1)

To report SUSPECTED ADVERSE REACTIONS, contact Amgen Inc. at 1-877-Nplate1 (1-877-675-2831) or FDA at 1-800-FDA-1088 or www.fda.gov/medwatch.

----------USE IN SPECIFIC POPULATIONS----------

- Pregnancy: Based on animal data, Nplate may cause fetal harm. Enroll pregnant patients in the Nplate pregnancy registry by calling 1-877-Nplate1 (1-877-675-2831). (8.1)
- Nursing Mothers: A decision should be made to discontinue Nplate or nursing, taking into account the importance of Nplate to the mother. (8.3)

See 17 FOR PATIENT COUNSELING INFORMATION AND MEDICATION GUIDE.

Revised: 08/2008

FULL PRESCRIBING INFORMATION: CONTENTS*

*Sections or subsections omitted from the full prescribing information are not listed.

FULL PRESCRIBING INFORMATION

1 INDICATIONS AND USAGE

Nplate is indicated for the treatment of thrombocytopenia in patients with chronic immune (idiopathic) thrombocytopenic purpura (ITP) who have had an insufficient response to corticosteroids, immunoglobulins or splenectomy. Nplate should be used only in patients with ITP whose degree of thrombocytopenia and clinical condition increases the risk for bleeding. Nplate should not be used in an attempt to normalize platelet counts.

2 DOSAGE AND ADMINISTRATION

Only prescribers enrolled in the Nplate NEXUS (Network of Experts Understanding and Supporting Nplate and Patients) Program may prescribe Nplate [*see Warnings and Precautions (5.7)*]. Nplate must be administered by the enrolled prescribers or healthcare providers under their direction.

2.1 Recommended Dosage Regimen

Monitor complete blood counts (CBCs), including platelet counts and peripheral blood smears, prior to initiation of Nplate and throughout Nplate therapy. Monitor CBCs, including platelet counts, for at least 2 weeks following discontinuation of Nplate [*see Warnings and Precautions (5.6)*].

Use the lowest dose of Nplate to achieve and maintain a platelet count $\geq 50 \times 10^9$/L as necessary to reduce the risk for bleeding. Administer Nplate as a weekly subcutaneous injection with dose adjustments based upon the platelet count response. Nplate should not be used in an attempt to normalize platelet counts [*see Warnings and Precautions (5.3)*].

The prescribed Nplate dose may consist of a very small volume (eg, 0.15 mL). Administer Nplate only with a syringe that contains 0.01 mL graduations.

Initial Dose
The initial dose for Nplate is 1 mcg/kg based on actual body weight.

Dose Adjustments
Use the actual body weight at initiation of therapy, then adjust the weekly dose of Nplate by increments of 1 mcg/kg until the patient achieves a platelet count $\geq 50 \times 10^9$/L as necessary to reduce the risk for bleeding; do not exceed a maximum weekly dose of 10 mcg/kg. In clinical studies, most patients who responded to Nplate achieved and maintained platelet counts $\geq 50 \times 10^9$/L with a median dose of 2 mcg/kg.

During Nplate therapy, assess CBCs, including platelet count and peripheral blood smears, weekly until a stable platelet count ($\geq 50 \times 10^9$/L for at least 4 weeks without dose adjustment) has been achieved. Obtain CBCs, including platelet counts and peripheral blood smears, monthly thereafter.

Adjust the dose as follows:

- If the platelet count is $< 50 \times 10^9$/L, increase the dose by 1 mcg/kg.
- If platelet count is $> 200 \times 10^9$/L for 2 consecutive weeks, reduce the dose by 1 mcg/kg.
- If platelet count is $> 400 \times 10^9$/L, do not dose. Continue to assess the platelet count weekly. After the platelet count has fallen to $< 200 \times 10^9$/L, resume Nplate at a dose reduced by 1 mcg/kg.

Discontinuation
Discontinue Nplate if the platelet count does not increase to a level sufficient to avoid clinically important bleeding after 4 weeks of Nplate therapy at the maximum weekly dose of 10 mcg/kg [*see Warnings and Precautions (5.4)*]. Obtain CBCs, including platelet counts, weekly for at least 2 weeks following discontinuation of Nplate [*see Warnings and Precautions (5.6)*].

2.2 Preparation and Administration

Nplate is supplied in single-use vials as a sterile, preservative-free, white lyophilized powder that must be reconstituted as outlined in Table 1 and administered using a syringe with 0.01 mL graduations. Using aseptic technique, reconstitute Nplate with preservative-free Sterile Water for Injection, USP as described in Table 1. Do not use bacteriostatic water for injection.

Table 1. Reconstitution of Nplate Single-Use Vials

Nplate Single-Use Vial	Total Vial Content of Romiplostim		Sterile Water for Injection*		Deliverable Product and Volume	Final Concentration
250 mcg	375 mcg	add	0.72 mL	=	250 mcg in 0.5 mL	500 mcg/mL
500 mcg	625 mcg	add	1.2 mL	=	500 mcg in 1 mL	500 mcg/mL

* Use preservative-free Sterile Water for Injection.

Gently swirl and invert the vial to reconstitute. Avoid excess or vigorous agitation: **DO NOT SHAKE**. Generally, dissolution of Nplate takes less than 2 minutes. The reconstituted Nplate solution should be clear and colorless. Visually inspect the reconstituted solution for particulate matter and/or discoloration. Do not administer Nplate if particulate matter and/or discoloration is observed.

Reconstituted Nplate can be kept at room temperature (25°C/77°F) or refrigerated at 2° to 8°C (36° to 46°F) for up to 24 hours prior to administration. Protect the reconstituted product from light.

To determine the injection volume to be administered, first identify the patient's total dose in micrograms (mcg) using the dosing information in Section 2.1. For example, a 75 kg patient initiating therapy at 1 mcg/kg will begin with a dose of 75 mcg. Next, calculate the volume of Nplate solution that is given to the patient by dividing the microgram dose by the concentration of the reconstituted Nplate solution (500 mcg/mL). For this patient example, the 75 mcg dose is divided by 500 mcg/mL, resulting in an injection volume of 0.15 mL.

As the injection volume may be very small, use a syringe with graduations to 0.01 mL.

Discard any unused portion. Do not pool unused portions from the vials. Do not administer more than one dose from a vial.

2.3 Use of Nplate With Concomitant Medical ITP Therapies

Nplate may be used with other medical ITP therapies, such as corticosteroids, danazol, azathioprine, intravenous immunoglobulin (IVIG), and anti-D immunoglobulin. If the patient's platelet count is $\geq 50 \times 10^9$/L, medical ITP therapies may be reduced or discontinued [*see Clinical Studies (14.1)*].

3 DOSAGE FORMS AND STRENGTHS

Single-use vials contain 250 or 500 mcg of deliverable romiplostim as a sterile, lyophilized, solid white powder.

4 CONTRAINDICATIONS

None

5 WARNINGS AND PRECAUTIONS

5.1 Bone Marrow Reticulin Formation and Risk for Bone Marrow Fibrosis

Nplate administration increases the risk for development or progression of reticulin fiber deposition within the bone marrow. In clinical studies, Nplate was discontinued in four of the 271 patients because of bone marrow reticulin deposition. Six additional patients had reticulin observed upon bone marrow biopsy. All 10 patients with bone marrow reticulin deposition had received Nplate doses $\geq$ 5 mcg/kg and six received doses $\geq$ 10 mcg/kg. Progression

to marrow fibrosis with cytopenias was not reported in the controlled clinical studies. In the extension study, one patient with ITP and hemolytic anemia developed marrow fibrosis with collagen during Nplate therapy. Clinical studies have not excluded a risk of bone marrow fibrosis with cytopenias.

Prior to initiation of Nplate, examine the peripheral blood smear closely to establish a baseline level of cellular morphologic abnormalities. Following identification of a stable Nplate dose, examine peripheral blood smears and CBCs monthly for new or worsening morphological abnormalities (eg, teardrop and nucleated red blood cells, immature white blood cells) or cytopenia(s). If the patient develops new or worsening morphological abnormalities or cytopenia(s), discontinue treatment with Nplate and consider a bone marrow biopsy, including staining for fibrosis [*see Adverse Reactions (6.1)*].

5.2 Worsened Thrombocytopenia After Cessation of Nplate

Discontinuation of Nplate may result in thrombocytopenia of greater severity than was present prior to Nplate therapy. This worsened thrombocytopenia may increase the patient's risk of bleeding, particularly if Nplate is discontinued while the patient is on anticoagulants or antiplatelet agents. In clinical studies of patients with chronic ITP who had Nplate discontinued, four of 57 patients developed thrombocytopenia of greater severity than was present prior to Nplate therapy. This worsened thrombocytopenia resolved within 14 days. Following discontinuation of Nplate, obtain weekly CBCs, including platelet counts, for at least 2 weeks and consider alternative treatments for worsening thrombocytopenia, according to current treatment guidelines [*see Adverse Reactions (6.1)*].

5.3 Thrombotic/Thromboembolic Complications

Thrombotic/thromboembolic complications may result from excessive increases in platelet counts. Excessive doses of Nplate or medication errors that result in excessive Nplate doses may increase platelet counts to a level that produces thrombotic/thromboembolic complications. In controlled clinical studies, the incidence of thrombotic/thromboembolic complications was similar between Nplate and placebo. To minimize the risk for thrombotic/thromboembolic complications, do not use Nplate in an attempt to normalize platelet counts. Follow the dose adjustment guidelines to achieve and maintain a platelet count of $\geq 50 \times 10^9$/L [*see Dosage and Administration (2.1)*].

5.4 Lack or Loss of Response to Nplate

Hyporesponsiveness or failure to maintain a platelet response with Nplate should prompt a search for causative factors, including neutralizing antibodies to Nplate or bone marrow fibrosis [*see Warnings and Precautions (5.1) and Adverse Reactions (6.2)*]. To detect antibody formation, submit blood samples to Amgen (1-800-772-6436). Amgen will assay these samples for antibodies to Nplate and thrombopoietin (TPO). Discontinue Nplate if the platelet count does not increase to a level sufficient to avoid clinically important bleeding after 4 weeks at the highest weekly dose of 10 mcg/kg.

5.5 Malignancies and Progression of Malignancies

Nplate stimulation of the TPO receptor on the surface of hematopoietic cells may increase the risk for hematologic malignancies. In controlled clinical studies among patients with chronic ITP, the incidence of hematologic malignancy was low and similar between Nplate and placebo. In a separate single-arm clinical study of 44 patients with myelodysplastic syndrome (MDS), 11 patients were reported as having possible disease progression, among whom four patients had confirmation of acute myelogenous leukemia (AML) during follow-up. Nplate is not indicated for the treatment of thrombocytopenia due to MDS or any cause of thrombocytopenia other than chronic ITP.

5.6 Laboratory Monitoring

Monitor CBCs, including platelet counts and peripheral blood smears, prior to initiation, throughout, and following discontinuation of Nplate therapy. Prior to the initiation of Nplate, examine the peripheral blood differential to establish the baseline extent of red and white blood cell abnormalities. Obtain CBCs, including platelet counts and

peripheral blood smears, weekly during the dose adjustment phase of Nplate therapy and then monthly following establishment of a stable Nplate dose. Obtain CBCs, including platelet counts, weekly for at least 2 weeks following discontinuation of Nplate [*see Dosage and Administration (2.1) and Warnings and Precautions (5.1, 5.2)*].

5.7 Nplate Distribution Program

Nplate is available only through a restricted distribution program called Nplate NEXUS (Network of Experts Understanding and Supporting Nplate and Patients) Program. Under the Nplate NEXUS Program, only prescribers and patients registered with the program are able to prescribe, administer, and receive Nplate. This program provides educational materials and a mechanism for the proper use of Nplate. To enroll in the Nplate NEXUS Program, call 1-877-Nplate1 (1-877-675-2831). Prescribers and patients are required to understand the risks of Nplate therapy. Prescribers are required to understand the information in the prescribing information and be able to:

- Educate patients on the benefits and risks of treatment with Nplate, ensure that the patient receives the Medication Guide, instruct them to read it, and encourage them to ask questions when considering Nplate. Patients may be educated by the enrolled prescriber or a healthcare provider under that prescriber's direction.
- Review the Nplate NEXUS Program Healthcare Provider Enrollment Form, sign the form, and return the form according to Nplate NEXUS Program instructions.
- Review the Nplate NEXUS Program Patient Enrollment Form, answer all questions, obtain the patient's signature on the Nplate NEXUS Program Patient Enrollment Form, place the original signed form in the patient's medical record, send a copy according to Nplate NEXUS Program instructions, and give a copy to the patient.
- Report any serious adverse events associated with the use of Nplate to the Nplate NEXUS Program Call Center at 1-877-Nplate1 (1-877-675-2831) or to the FDA's MedWatch Program at 1-800-FDA-1088.
- Report serious adverse events observed in patients receiving Nplate, including events actively solicited at 6-month intervals.

6 ADVERSE REACTIONS

6.1 Clinical Studies Experience

Serious adverse reactions associated with Nplate in clinical studies were bone marrow reticulin deposition and worsening thrombocytopenia after Nplate discontinuation [*see Warnings and Precautions (5.1, 5.2)*].

The data described below reflect Nplate exposure to 271 patients with chronic ITP, aged 18 to 88, of whom 62% were female. Nplate was studied in two randomized, placebo-controlled, double-blind studies that were identical in design, with the exception that Study 1 evaluated nonsplenectomized patients with ITP and Study 2 evaluated splenectomized patients with ITP. Data are also reported from an open-label, single-arm study in which patients received Nplate over an extended period of time. Overall, Nplate was administered to 114 patients for at least 52 weeks and 53 patients for at least 96 weeks.

Because clinical studies are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not reflect the rates observed in practice.

In the placebo-controlled studies, headache was the most commonly reported adverse drug reaction, occurring in 35% of patients receiving Nplate and 32% of patients receiving placebo. Headaches were usually of mild or moderate severity. Table 2 presents adverse drug reactions from Studies 1 and 2 with a $\geq$ 5% higher patient incidence in Nplate versus placebo. The majority of these adverse drug reactions were mild to moderate in severity.

Table 2. Adverse Drug Reactions Identified in Two Placebo-Controlled Studies

Preferred Term	Nplate (n = 84)	Placebo (n = 41)
Arthralgia	26%	20%
Dizziness	17%	0%
Insomnia	16%	7%
Myalgia	14%	2%
Pain in Extremity	13%	5%
Abdominal Pain	11%	0%
Shoulder Pain	8%	0%
Dyspepsia	7%	0%
Paresthesia	6%	0%

Among 142 patients with chronic ITP who received Nplate in the single-arm extension study, the incidence rates of the adverse reactions occurred in a pattern similar to those reported in the placebo-controlled clinical studies.

6.2 Immunogenicity

As with all therapeutic proteins, patients may develop antibodies to the therapeutic protein. Patients were screened for immunogenicity to romiplostim using a BIAcore-based biosensor immunoassay. This assay is capable of detecting both high- and low-affinity binding antibodies that bind to romiplostim and cross-react with TPO. The samples from patients that tested positive for binding antibodies were further evaluated for neutralizing capacity using a cell-based bioassay.

In clinical studies, the incidence of preexisting antibodies to romiplostim was 8% (17/225) and the incidence of binding antibody development during Nplate treatment was 10% (23/225). The incidence of preexisting antibodies to endogenous TPO was 5% (12/225) and the incidence of binding antibody development to endogenous TPO during Nplate treatment was 5% (12/225). Of the patients with positive antibodies to romiplostim or to TPO, one (0.4%) patient had neutralizing activity to romiplostim and none had neutralizing activity to TPO. No correlation was observed between antibody activity and clinical effectiveness or safety.

Immunogenicity assay results are highly dependent on the sensitivity and specificity of the assay used in detection and may be influenced by several factors, including sample handling, concomitant medications, and underlying disease. For these reasons, comparison of incidence of antibodies to romiplostim with the incidence of antibodies to other products may be misleading.

7 DRUG INTERACTIONS

No formal drug interaction studies of Nplate have been performed.

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy

Pregnancy Category C
There are no adequate and well-controlled studies of Nplate use in pregnant women. In animal reproduction and developmental toxicity studies, romiplostim crossed the placenta, and adverse fetal effects included thrombocytosis, postimplantation loss, and an increase in pup mortality. Nplate should be used during pregnancy only if the potential benefit to the mother justifies the potential risk to the fetus.

Pregnancy Registry: A pregnancy registry has been established to collect information about the effects of Nplate use during pregnancy. Physicians are encouraged to register pregnant patients, or pregnant women may enroll themselves in the Nplate pregnancy registry by calling 1-877-Nplate1 (1-877-675-2831).

In rat and rabbit developmental toxicity studies no evidence of fetal harm was observed at romiplostim doses up to 11 times (rats) and 82 times (rabbit) the maximum human dose (MHD) based on systemic exposure. In mice at doses 5 times the MHD, reductions in maternal body weight and increased postimplantation loss occurred.

In a prenatal and postnatal development study in rats, at doses 11 times the MHD, there was an increase in perinatal pup mortality. Romiplostim crossed the placental barrier in rats and increased fetal platelet counts at clinically equivalent and higher doses.

8.3 Nursing Mothers

It is not known whether Nplate is excreted in human milk; however, human IgG is excreted in human milk. Published data suggest that breast milk antibodies do not enter the neonatal and infant circulation in substantial amounts. Because many drugs are excreted in human milk and because of the potential for serious adverse reactions in nursing infants from Nplate, a decision should be made whether to discontinue nursing or to discontinue Nplate, taking into account the importance of Nplate to the mother and the known benefits of nursing.

8.4 Pediatric Use

The safety and effectiveness in pediatric patients (< 18 years) have not been established.

8.5 Geriatric Use

Of the 271 patients who received Nplate in ITP clinical studies, 55 (20%) were age 65 and over, and 27 (10%) were 75 and over. No overall differences in safety or efficacy have been observed between older and younger patients in the placebo-controlled studies, but greater sensitivity of some older individuals cannot be ruled out. In general, dose adjustment for an elderly patient should be cautious, reflecting the greater frequency of decreased hepatic, renal, or cardiac function, and of concomitant disease or other drug therapy.

8.6 Renal Impairment

No clinical studies were conducted in patients with renal impairment. Use Nplate with caution in this population.

8.7 Hepatic Impairment

No clinical studies were conducted in patients with hepatic impairment. Use Nplate with caution in this population.

10 OVERDOSAGE

In the event of overdose, platelet counts may increase excessively and result in thrombotic/thromboembolic complications. In this case, discontinue Nplate and monitor platelet counts. Reinitiate treatment with Nplate in accordance with dosing and administration recommendations [*see Dosage and Administration (2.2)*].

11 DESCRIPTION

Romiplostim, a member of the TPO mimetic class, is an Fc-peptide fusion protein (peptibody) that activates intracellular transcriptional pathways leading to increased platelet production via the TPO receptor (also known as cMpl). The peptibody molecule contains two identical single-chain subunits, each consisting of human immunoglobulin IgG1 Fc domain, covalently linked at the C-terminus to a peptide containing two thrombopoietin receptor-binding domains. Romiplostim has no amino acid sequence homology to endogenous TPO. Romiplostim is produced by recombinant DNA technology in *Escherichia coli (E coli)*.

Nplate is supplied as a sterile, preservative-free, lyophilized, solid white powder for subcutaneous injection. Two vial presentations are available, which contain a sufficient amount of active ingredient to provide either 250 mcg or 500 mcg of deliverable romiplostim, respectively. Each single-use 250 mcg vial of Nplate contains the following: 375 mcg romiplostim, 30 mg mannitol, 15 mg sucrose, 1.2 mg L-histidine, 0.03 mg polysorbate 20, and sufficient HCl to adjust the pH to a target of 5.0. Each single-use 500 mcg vial of Nplate contains the following: 625 mcg romiplostim, 50 mg mannitol, 25 mg sucrose, 1.9 mg L-histidine, 0.05 mg polysorbate 20, and sufficient HCl to adjust the pH to a target of 5.0 [*see Dosage and Administration (2.2)*].

12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action

Nplate increases platelet production through binding and activation of the TPO receptor, a mechanism analogous to endogenous TPO.

12.2 Pharmacodynamics

In clinical studies, treatment with Nplate resulted in dose-dependent increases in platelet counts. After a single subcutaneous dose of 1 to 10 mcg/kg Nplate in patients with chronic ITP, the peak platelet count was 1.3 to 14.9 times greater than the baseline platelet count over a 2- to 3-week period. The platelet counts were above 50 x 10^9/L for seven out of eight patients with chronic ITP who received six weekly doses of Nplate at 1 mcg/kg.

12.3 Pharmacokinetics

In the long-term extension study in patients with ITP receiving weekly treatment of Nplate subcutaneously, the pharmacokinetics of romiplostim over the dose range of 3 to 15 mcg/kg indicated that peak serum concentrations of romiplostim were observed about 7 to 50 hours post dose (median: 14 hours) with half-life values ranging from 1 to 34 days (median: 3.5 days). The serum concentrations varied among patients and did not correlate with the dose administered. The elimination of serum romiplostim is in part dependent on the TPO receptor on platelets. As a result, for a given dose, patients with high platelet counts are associated with low serum concentrations and vice versa. In another ITP clinical study, no accumulation in serum concentrations was observed (n = 4) after six weekly doses of Nplate (3 mcg/kg). The accumulation at higher doses of romiplostim is unknown.

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

The carcinogenic potential of romiplostim has not been evaluated. The mutagenic potential of romiplostim has not been evaluated. Romiplostim had no effect on the fertility of rats at doses up to 37 times the MHD based on systemic exposure.

13.2 Animal Toxicology and/or Pharmacology

In a 4-week repeat-dose toxicity study in which rats were dosed subcutaneously three times per week, romiplostim caused extramedullary hematopoiesis, bone hyperostosis and marrow fibrosis at clinically equivalent and higher doses. In this study, these findings were not observed in animals after a 4-week post treatment recovery period. Studies of long-term treatment with romiplostim in rats have not been conducted; therefore, it is not known if the fibrosis of the bone marrow is reversible in rats after long-term treatment.

14 CLINICAL STUDIES

14.1 Chronic ITP

The safety and efficacy of Nplate were assessed in two double-blind, placebo-controlled clinical studies and in an open-label extension study.

Studies 1 and 2

In Studies 1 and 2, patients with chronic ITP who had completed at least one prior treatment and had a platelet count of $\leq 30 \times 10^9$/L prior to study entry were randomized (2:1) to 24 weeks of Nplate (1 mcg/kg subcutaneous [SC]) or placebo. Prior ITP treatments in both study groups included corticosteroids, immunoglobulins, rituximab, cytotoxic therapies, danazol, and azathioprine. Patients already receiving ITP medical therapies at a constant dosing schedule were allowed to continue receiving these medical treatments throughout the studies. Rescue therapies (ie, corticosteroids, IVIG, platelet transfusions, and anti-D immunoglobulin) were permitted for bleeding, wet purpura, or if the patient was at immediate risk for hemorrhage. Patients received single weekly SC injections of Nplate, with individual dose adjustments to maintain platelet counts (50×10^9/L to 200×10^9/L).

Study 1 evaluated patients who had not undergone a splenectomy. The patients had been diagnosed with ITP for approximately 2 years and had received a median of three prior ITP treatments. Overall, the median platelet count was 19×10^9/L at study entry. During the study, the median weekly Nplate dose was 2 mcg/kg (25th–75th percentile: 1–3 mcg/kg).

Study 2 evaluated patients who had undergone a splenectomy. The patients had been diagnosed with ITP for approximately 8 years and had received a median of six prior ITP treatments. Overall, the median platelet count was 14×10^9/L at study entry. During the study, the median weekly Nplate dose was 3 mcg/kg (25th–75th percentile: 2–7 mcg/kg).

Study 1 and 2 outcomes are shown in Table 3. A durable platelet response was the achievement of a weekly platelet count $\geq 50 \times 10^9$/L for any 6 of the last 8 weeks of the 24-week treatment period in the absence of rescue medication at any time. A transient platelet response was the achievement of any weekly platelet counts $\geq 50 \times 10^9$/L for any 4 weeks during the treatment period without a durable platelet response. An overall platelet response was the achievement of either a durable or a transient platelet response. Platelet responses were excluded for 8 weeks after receiving rescue medications.

Table 3. Results From Placebo-Controlled Studies[a]

Outcomes	Study 1 Nonsplenectomized Patients		Study 2 Splenectomized Patients	
	Nplate (n = 41)	Placebo (n = 21)	Nplate (n = 42)	Placebo (n = 21)
Platelet Responses and Rescue Therapy				
Durable Platelet Response, n (%)	25 (61%)	1 (5%)	16 (38%)	0 (0%)
Overall Platelet Response, n (%)	36 (88%)	3 (14%)	33 (79%)	0 (0%)
Number of Weeks With Platelet Counts $\geq 50 \times 10^9$/L, average	15	1	12	0
Requiring Rescue Therapy, n (%)	8 (20%)	13 (62%)	11 (26%)	12 (57%)
Reduction/Discontinuation of Baseline Concurrent ITP Medical Therapy				
Receiving Therapy at Baseline	(n = 11)	(n = 10)	(n = 12)	(n = 6)
Patients Who Had > 25% Dose Reduction in Concurrent Therapy, n (%)	4/11 (36%)	2/10 (20%)	4/12 (33%)	1/6 (17%)
Patients Who Discontinued Baseline Therapy, n (%)[b]	4/11 (36%)	3/10 (30%)	8/12 (67%)	0/6 (0%)

[a] All *P* values < 0.05 for platelet response and rescue therapy comparisons between Nplate and placebo.

[b] For multiple concomitant baseline therapies, all therapies were discontinued.

In Studies 1 and 2, nine patients reported a serious bleeding event [five (6%) Nplate, four (10%) placebo]. Bleeding events that were grade 2 severity or higher occurred in 15% of patients treated with Nplate and 34% of patients treated with placebo.

Extension Study
Patients who had participated in either Study 1 or Study 2 were withdrawn from study medications. If platelet counts subsequently decreased to $\leq 50 \times 10^9$/L, the patients were allowed to receive Nplate in an open-label extension study with weekly dosing based on platelet counts. Following Nplate discontinuation in Studies 1 and 2, seven patients maintained platelet counts of $\geq 50 \times 10^9$/L. Among 100 patients who subsequently entered the extension study, platelet counts were increased and sustained regardless of whether they had received Nplate or placebo in the prior placebo-controlled studies. The majority of patients reached a median platelet count of 50×10^9/L after receiving one to three doses of Nplate, and these platelet counts were maintained throughout the remainder of the study with a median duration of Nplate treatment of 60 weeks and a maximum duration of 96 weeks.

16 HOW SUPPLIED/STORAGE AND HANDLING

Nplate is supplied in single-use vials containing 250 mcg (NDC 55513-221-01) and 500 mcg (NDC 55513-222-01) deliverable romiplostim.

Store Nplate vials in their carton to protect from light until time of use. Keep Nplate vials refrigerated at 2° to 8°C (36° to 46°F). Do not freeze.

17 PATIENT COUNSELING INFORMATION

See FDA-Approved Medication Guide.

17.1 Information for Patients

Prior to treatment, patients should fully understand the risks and benefits of Nplate. Inform patients that the risks associated with long-term administration of Nplate are unknown and that they must enroll in the Nplate NEXUS Program, which provides for the proper use of Nplate in ITP patients.

Inform patients of the following risks and considerations for Nplate:

- Nplate can only be administered by a healthcare provider who is enrolled in the Nplate NEXUS Program or a healthcare provider under their direction.
- Nplate therapy is administered to achieve and maintain a platelet count $\geq 50 \times 10^9$/L as necessary to reduce the risk for bleeding; Nplate is not used to normalize platelet counts.
- Following discontinuation of Nplate, thrombocytopenia and risk of bleeding may develop that is worse than that experienced prior to the Nplate therapy.
- Nplate therapy increases the risk of reticulin fiber formation within the bone marrow, and further fiber formation may progress to marrow fibrosis. Detection of peripheral blood cell abnormalities may necessitate a bone marrow examination.
- Too much Nplate may result in excessive platelet counts and a risk for thrombotic/thromboembolic complications.
- Nplate stimulates certain bone marrow cells to make platelets and may increase the risk for progression of underlying MDS or hematological malignancies.
- Platelet counts and CBCs, including peripheral blood smears, must be performed weekly until a stable Nplate dose has been achieved; thereafter, platelet counts and CBCs, including peripheral blood smears, must be performed monthly while taking Nplate.
- Patients must be closely monitored with weekly platelet counts and CBCs for at least 2 weeks following Nplate discontinuation.
- Even with Nplate therapy, patients should continue to avoid situations or medications that may increase the risk for bleeding.

17.2 FDA-Approved Medication Guide

AMGEN®

Nplate™ (romiplostim)

Manufactured by:
Amgen Inc.
One Amgen Center Drive
Thousand Oaks, California 91320-1799

This product, its production, and/or its use may be covered by one or more U.S. Patents, including U.S. Patent Nos. 6,835,809 and 7,189,827, as well as other patents or patents pending.

www.Nplate.com
1-877-Nplate1 (1-877-675-2831)

3xxxxx
v1

MEDICATION GUIDE

Nplate™ (N-plāt)

(romiplostim)

Read this Medication Guide before you start Nplate and before each Nplate injection. There may be new information. This Medication Guide does not take the place of talking to your healthcare provider about your medical condition or your treatment.

What is the most important information I should know about Nplate?

Nplate can cause uncommon but serious side effects:

- **Bone marrow changes (increased reticulin and possible bone marrow fibrosis).** Long-term use of Nplate may cause changes in your bone marrow. These changes may lead to abnormal blood cells or your body making less blood cells. The mild form of these bone marrow changes is called "increased reticulin." It is not known if this may progress to a more severe form called "fibrosis." The mild form may cause no problems while the severe form may cause life-threatening blood problems. Signs of bone marrow changes may show up as abnormalities in your blood tests. Your healthcare provider will decide if abnormal blood tests mean that you should have bone marrow tests or if you should stop taking Nplate.

- **Worsening low blood platelet count (thrombocytopenia) and risk of bleeding shortly after stopping Nplate.** When you stop receiving Nplate, your low blood platelet count (thrombocytopenia) may become worse than before you started receiving Nplate. These effects are most likely to happen shortly after stopping Nplate and may last about 2 weeks. The lower platelet counts during this time period may increase your risk of bleeding, especially if you are taking a blood thinner or other medicine that affects platelets. Your healthcare provider will check your blood platelet counts for at least two weeks after you stop taking Nplate. Call your healthcare provider right away to report any bruising or bleeding.

- **High platelet counts and higher chance for blood clots.** You have a higher chance of getting a blood clot if your platelet count is too high during treatment with Nplate. You may have severe complications or die from some forms of blood clots, such as clots that spread to the lungs or that cause heart attacks or strokes. Your healthcare provider will check your blood platelet counts and change your dose or stop Nplate if your platelet counts get too high.

- **Worsening of blood cancers.** Nplate is not for use in patients with blood cancer or a precancerous condition called myelodysplastic syndrome (MDS). If you have one of these conditions, Nplate may worsen your cancer or condition and may cause you to die sooner.

When you are being treated with Nplate, your healthcare provider will closely monitor your Nplate dose and blood tests, including platelet counts.

- Nplate is available only after you and your healthcare provider agree to join a program that is intended to help in the safe use of Nplate. This program is called the "Nplate NEXUS Program."

- Only a healthcare provider can inject a dose of Nplate. Injection of too much Nplate may cause a dangerous increase in your blood platelet count and serious side effects.

- During Nplate therapy, your healthcare provider may change your Nplate dose, depending upon the change in your blood platelet count. You must have blood platelet counts done before you start Nplate, during Nplate therapy, and after Nplate therapy is stopped.

- Nplate is used to try to keep your platelet count about 50,000 per microliter in order to lower the risk for bleeding. Nplate is not used to make your platelet count normal.

See "What are the possible side effects of Nplate?" for other side effects of Nplate.

What is Nplate?

Nplate is a man-made protein medicine used to treat low blood platelet counts in adults with chronic immune (idiopathic) thrombocytopenic purpura (ITP), when other medicine to treat your ITP is not the best choice for you or surgery to remove the spleen has not worked well enough.

Nplate is only:

- Prescribed by healthcare providers who are enrolled in the Nplate NEXUS Program.
- Given to patients who are enrolled in the Nplate NEXUS Program.
- Given by the enrolled healthcare provider or a provider under their direction. You may not give Nplate injections to yourself.

It is not known if Nplate works or if it is safe in people under the age of 18.

Nplate is for treatment of certain people with low blood platelet counts caused by chronic ITP, not low platelet counts caused by other conditions or diseases.

What should I tell my doctor before taking Nplate?

Tell your doctor about all your medical conditions, including if you:

- Have had surgery to remove your spleen (splenectomy).
- Have a bone marrow problem, including a blood cancer or MDS.
- Have or had a blood clot.
- Have bleeding problems.
- Are pregnant, think you may be pregnant, or plan to get pregnant. It is not known if Nplate will harm an unborn baby.

  Pregnancy Registry: There is a registry for women who become pregnant during treatment with Nplate. If you become pregnant, consider this registry. The purpose of the registry is to collect safety information about the health of you and your baby. Contact the registry as soon as you become aware of the pregnancy, or ask your healthcare provider to contact the registry for you. You or your healthcare provider can get information and enroll in the registry by calling 1-877-Nplatel (1-877-675-2831).

- Are breast-feeding or plan to breast-feed. It is not known if Nplate passes into your breast milk. You and your healthcare provider should decide whether you will take Nplate or breast-feed. You should not do both.

Tell your healthcare provider about all the medicines you take, including prescription and nonprescription medicines, vitamins, and herbal products. Know the medicines you take. Keep a list of them and show it to your healthcare provider and pharmacist when you get a new medicine.

How should I take Nplate?

To receive Nplate, you must first talk with your healthcare provider and understand the benefits and risks of Nplate. You must agree to and follow all of the instructions in the Nplate NEXUS Program.

Before you can begin to receive Nplate, your healthcare provider will:

- Explain the Nplate NEXUS Program to you.
- Answer all of your questions about Nplate and the Nplate NEXUS Program.
- Make sure you read the Nplate Medication Guide.
- Have you sign the Nplate NEXUS Patient Enrollment Form.

Nplate is given by your healthcare provider as a subcutaneous (SC) injection under the skin one time each week.

Your healthcare provider will check your platelet count every week and change your dose of Nplate as needed. This will continue until your healthcare provider decides that your dose of Nplate can stay the same. After that, you will need to have blood tests every month. When you stop receiving Nplate, you will need blood tests for at least 2 weeks to check if your platelet count drops too low.

Tell your healthcare provider about any bruising or bleeding that occurs while you are receiving Nplate.

If you miss a scheduled dose of Nplate, call your healthcare provider to arrange for your next dose as soon as possible.

What should I avoid while receiving Nplate?

Avoid situations that may increase your risk of bleeding, such as missing a scheduled dose of Nplate. You should arrange for your next dose as soon as possible. Call your doctor or the Nplate NEXUS Program at 1-877-Nplatel (1-877-675-2831).

What are the possible side effects of Nplate?

Nplate may cause serious side effects. See **"What is the most important information I should know about Nplate?"**

The most common side effects of Nplate are:

- Headache
- Joint pain
- Dizziness
- Trouble sleeping
- Muscle tenderness or weakness
- Pain in arms and legs
- Abdominal pain
- Shoulder pain
- Indigestion
- Tingling or numbness in hands and feet

These are not all the possible side effects of Nplate. Tell your healthcare provider if you have any side effect that bothers you or that does not go away. For more information, ask your healthcare provider or pharmacist.

Call your doctor for medical advice about side effects. You may report side effects to the Nplate NEXUS Program at 1-877-Nplatel (1-877-675-2831) or FDA at 1-800-FDA-1088.

General information about the safe and effective use of Nplate.

This Medication Guide summarizes the most important information about Nplate. If you would like more information, talk with your healthcare provider. You can ask your healthcare provider or pharmacist for information about Nplate that is written for health professionals.

What are the ingredients in Nplate?

Active ingredient: romiplostim
Inactive ingredients: L-histidine, sucrose, mannitol, polysorbate 20, and hydrochloric acid

Nplate™ (romiplostim)
Manufactured by:
Amgen Inc.
One Amgen Center Drive
Thousand Oaks, California 91320-1799

This product, its production, and/or its use may be covered by one or more U.S. Patents, including U.S. Patent Nos. 6,835,809 and 7,189,827, as well as other patents or patents pending.

www.Nplate.com
1-877-Nplate1 (1-877-675-2831)

3xxxxxx
v1 Issue Date: 08/2008

This Medication Guide has been approved by the U.S. Food and Drug Administration.